Investigation into the dietary determinants of heart rate variability and potential mechanisms

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King's College London

INVESTIGATION INTO THE DIETARY DETERMINANTS OF HEART RATE VARIABILITY AND POTENTIAL MECHANISMS

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A thesis submitted to King's College London for the degree of Doctor of Philosophy in the Faculty of Science

Diabetes and Nutritional Sciences Division

School of Medicine

King's College London

September 2017

Publications, conference presentations, prizes and travel grants

Publications

Pinto AM, Al-Khatib H, Sanders TAB, Hall WL. Heart rate variability and long chain n-3 PUFA dietary intake and status: a comparison between matched vegans and omnivores. Proceedings of the Nutrition Society. 2015; 74(OCE5): E307.

Pinto AM, Sanders TAB, Kendall AC, Nicolaou A, Gray R, Al-Khatib H, Hall WL. A comparison of heart rate variability, n-3 PUFA status and lipid mediator profile in age- and BMI-matched middle-aged vegans and omnivores. Br J Nutr. 2017;117(5):669–85.

Pinto A, Bordoli C, Buckner L, Kaplan P, Arenal I, Jeffcock E, Kim C, Johnston K, Hall W. A randomised controlled trial assessing the impact of intermittent vs continuous energy restriction on blood pressure and anthropometry in healthy subjects with central obesity. "The Met-IER study". Obesity facts 2017;10(suppl 1):1-259.

Pinto A, Bordoli C, Buckner L, Kaplan P, Arenal I, Jeffcock E, Kim C, Johnston K, Hall W. A randomised controlled trial assessing the impact of intermittent energy restriction (IER) on weight loss and insulin sensitivity in healthy men and women with central obesity. "The Met-IER study". Obesity facts. 2017;10(suppl 1):1-259.

Conference presentations

Pinto AM, Sanders TAB, Hall WL. Effect of low doses of long chain n-3 PUFA intake on daytime heart rate variability: results from the MARINA study. EuroPRevent 2015, Lisbon, Portugal (poster presentation).

Pinto AM, Al-Khatib H, Sanders TAB, Hall WL. Heart rate variability and long chain n-3 PUFA dietary intake and status: a comparison between matched vegans and omnivores. Nutrition Society summer meeting 2015, Newcastle, UK (oral presentation)

Pinto AM, Kendall A, Nicolaou A, Hall WL. Lipidomic analysis of plasma n-3 PUFA lipid mediators from vegans and omnivores. Lipids and Health: Risk, Reward and Revelation 2015, London, UK (poster presentation).

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Pinto A, Bordoli C, Buckner L, Kaplan P, Arenal I, Jeffcock E, Kim C, Johnston K, Hall W. A randomised controlled trial assessing the impact of intermittent energy restriction (IER) on weight loss and insulin sensitivity in healthy men and women with central obesity. "The Met-IER study". ECO 2017, Porto, Portugal (poster presentation T3P6)

Prizes and travel grants

EJLST poster prize for best poster at Lipids and Health: Risk, Reward and Revelation, London, UK.

King's College London Graduate School Conference Grant 2017

Abstract

Heart rate variability (HRV), an indirect measure of cardiac autonomic function, is a powerful predictor of sudden cardiac death. Cardiac autonomic function is a neglected aspect of the pathophysiology of cardiovascular diseases (CVDs) in the field of nutritional sciences, with little published literature on effects of diet in this CVD risk factor. This thesis aimed at exploring the associations and impacts of some main dietary candidates that may influence HRV: long chain *n*-3 polyunsaturated fatty acids (LC *n*-3 PUFA) and energy intake restriction.

The first study compared HRV in vegans with omnivore controls, representing populations with low and high LC *n*-3 PUFA status, respectively. The observed differences between groups were more complex than hypothesised. Vegans presented higher 24 h overall HRV due to greater sleep-day differences compared to omnivores, and lower beat-to-beat HRV in the day-time. Vegans also presented lower availability of LC *n*-3 PUFA lipid mediators that may influence anti-inflammatory capacity, especially in populations with underlying chronic inflammation presenting low tissue LC *n*-3 PUFA status. This led to the next study, which characterised the variability of LC *n*-3 PUFA status in chronic kidney disease patients commencing haemodialysis, a population with high burden of inflammation and CVD compared to the general population, with the aim of establishing a potential relationship with HRV. Haemodialysis patients in this study had particularly low proportions and narrow degree of variability of erythrocyte membrane eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which precluded any meaningful findings in the relationship between tissue LC *n*-3 PUFA status and HRV. Finally, the last study compared the effects of intermittent and continuous energy restriction diets (IER vs. CER) on HRV in centrally obese, independent of weight loss. The results showed no superior effects of weight loss via an IER diet compared to a CER diet on HRV, and suggested that weight loss itself may have a much greater effect on HRV and other markers of cardio-metabolic risk than any independent effect of energy restriction type.

Author's contribution

The author was responsible for the planning of the studies incorporated in this doctoral research, including protocol writing, ethics submission, day-to-day running of the study days, blood sample processing, analysis of the data and writing of the thesis. The author performed the HRV analysis across all studies and supervised BSc, MSc and intercalated medical students that helped with running of the study days in the Om3ga and Met-IER studies. The author had the supervision, guidance and support from Dr. Wendy Hall across all the doctoral research, and Prof. Tom Sanders has also given guidance in the Om3ga study and also gave some valuable input to the FISHH study. Technician Robert Gray provided expertise and assisted in the GC analysis for the plasma and erythrocyte membrane fatty acid analysis in the Om3ga and FISHH studies. Although the author prepared the samples for the lipidomic analysis with the guidance of Dr. Alexandra Kendall, Dr. Kendall operated the LC/ESI-MS/MS machine as it required specific skills that were not within the scope of this PhD. The remaining blood biochemistry analyses were performed at the clinical biochemistry department at King's College Hospital (ViaPath) under the supervision of Tracy Dew.

Acknowledgements

I would like to thank everyone who has accompanied my PhD adventures over the last 5 years and made it possible for me to reach this far.

First, I would like to express how thankful I feel to have had Dr. Wendy Hall as my first supervisor, supporting me throughout all these years, providing me with the best guidance I could have wished for from a supervisor, allowing me to sail through the harder times and most of all, making me have an overall enjoyable experience as a PhD student. I would also like to thank Prof. Tom Sanders as my second supervisor for contributing with his expertise in the planning of the studies and the recruitment of vegan participants. Big thank you to Dr. Helen MacLaughlin for helping me with the FISHH study, from joining me to the ethics nhs review meeting, to the help recruiting patients and introducing me to the numerous members of the renal team staff across the KCH sites, and the valuable input in the renal study chapter. I would also like to thank Dr. Nicola Kumar and Dr. Jasmine Lee for all the help recruiting and introducing me to the renal team staff across the GSTT sites and letting me access the electronic medical records of the consented patients.

I would like to give a special thanks to Robert Gray for all his support in the lab with the fatty acids analysis, and to the other lab technicians Anne-Catherine Perz, Dave Lincoln and MJ Searle for their continuous support, and background work that made the study days always run as smooth as possible. A very special thanks to Rosie for all her help and patience helping me deal with the admin, ordering materials, and so many things of the day-to-day life as a PhD student. I am very grateful to Prof. Anna Nicolaou and Dr. Alexandra Kendall for the warm welcome to their department at the University of Manchester and for all their support with the lipidomic analysis.

Thank you so much to all the nurses from the renal staff team across all the KCH and GSTT units that collected the blood samples from the patients and for being so patient with the couriers that wouldn’t always do a straightforward job.

Thank you so much to all the volunteers of the studies, none of my doctoral research would have been possible without their goodwill to participate.

Very special thanks to my PhD office buddies Cathriona, Filomena, Monica, Angela and Haya that helped me so much in this journey, with extra special thanks to Haya for being my partner in crime through the write-up stage with all the joint work sessions and food delights to keep me motivated.

Thank you so much to Catherine, Haya, Luke, Polly, Claire, Curie, Ines and Emma, the best student crew I could have had to help with the day-to-day running of the PhD studies, making recruitment and study data collection a success, as well as being the best feel-good team ever! Thank you also for all the hard work put into going a step further from what you were initially set to.

Thank you to all my friends and family for your constant support and motivation. Special thanks to Ana Andreia and Anabela for all the baby sitting and the extra support and going so many extra miles to help me in so many ways.

Thank you to the end of the world to my parents, I don't even have the right words to use to express how grateful and lucky I am to have you as my parents. Thank you for all the support and giving me the chance to pursue my dreams, thank you for always being by my side and always going the extra mile to make me happy and help me in so many ways that if I were to state everything here would become another thesis!

Thank you to my other half Kelvin Sang, for finding me in the beginning of this PhD and making me the happiest human being, always giving me the support and motivation to make me thrive. Thank you for always making my work easier by sorting all the computers and laptops and creating the most amazing work conditions to write-up. Thank you for always enlightening me whenever I felt low and going the extra mile and doing the impossible to take care of Mia, especially in the couple of weeks preceding the submission of the thesis.

Leaving the most precious thank you to the end, thank you Mia for bringing so much joy to the last year of my PhD and for all the giggles and laughs and plays that kept me mentally balanced throughout the write-up stage.

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Abbreviations

AA	Arachidonic acid
ABP	Ambulatory blood pressure
ALA	α-linolenic acid
ANS	Autonomic nervous system
BHS	British hypertension society
BMI	Body mass index
bpm	beats per minute
Ca^{2+}	Calcium
CER	Continuous energy restriction
CHD	Coronary heart disease
CKD	Chronic kidney disease
CNS	Central nervous system
COX	Ciclooxygenase
cpm	Counts per minute
CRP	C-reactive protein
CVD	Cardiovascular disease
CYP	Cytochrome P450
DBP	Diastolic blood pressure
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
ECG	Electrocardiogram
EPA	Eicosapentaenoic acid
FFA3	Free fatty acid receptor 3
FFQ	Food frequency questionnaire
FFT	Fast Fourier transformation
GFR	Glomerular filtration rate

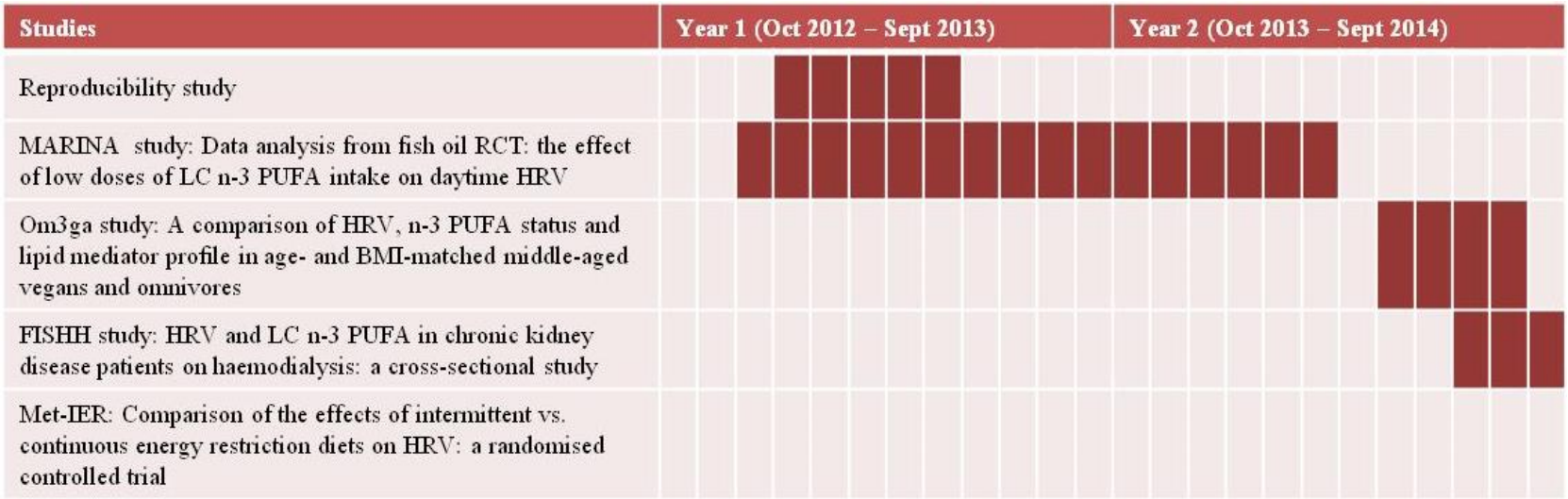
GPR41	G protein-coupled receptor 41
HDHA	Hydroxydocosahexaenoic acid
HDL	High density lipoprotein
HEPE	Hydroxyeicosapentaenoic acid
HF	High frequency
HR	Heart rate
HRV	Heart rate variability
hs-CRP	high sensitivity C-reactive protein
IBI	Interbeat interval
IER	Intermittent energy restriction
IL	Interleukin
K^+	Potassium
KCL	King's College London
LA	Linoleic acid
LC n-3 PUFA	Long chain n-3 polyunsaturated fatty acids
LDL	Low density lipoprotein
LF	Low frequency
LN	Natural logarithm
LOX	Lipoxygenase
MRU	Metabolic research unit
MST	Mnemonic similarity task
MUFA	Monounsaturated fatty acids
Na^+	Sodium
NN	Normal to normal
O3I	Omega-3 index
PG	Prostaglandin
pNN50	Percentage of adjacent NN intervals that differ by greater than 50 ms
PNS	Parasympathetic nervous system
PUFA	Polyunsaturated fatty acids

RCT	Randomised controlled trial
RMR	Resting metabolic rate
RMSSD	Root mean square of successive differences of NN intervals
Rv	Resolvin
SBP	Systolic blood pressure
SCD	Sudden cardiac death
SD	Standard deviation
SDNN	Standard deviations of NN intervals
SDANN	standard deviations of the average NN intervals
SER	Severe energy restriction
SNS	Sympathetic nervous system
SPM	Specialised pro-resolving mediators
TEE	Total energy expenditure
Ti	Triangular index
VLF	Very low frequency

Chapter 1 Introduction

The overarching scope of this thesis was to explore the role of diet and/or nutritional status in the prevention of sudden cardiac death (SCD) by investigating its impact on the autonomic nervous system (ANS) activity, assessed by heart rate variability (HRV), in healthy and diseased states. The effect of whole diets or dietary components on ANS function has rarely been considered and most of the available evidence derives from studies on energy balance and long chain n-3 polyunsaturated fatty acid (LC *n*-3 PUFA). This thesis will therefore explore how LC *n*-3 PUFA status could affect HRV by including an observational pilot study in a healthy population with low (vegans) and high (omnivores) LC *n*-3 PUFA status and a cross-sectional study in chronic kidney disease patients; and will explore how weight loss, either through continuous energy restriction or intermittent energy restriction could affect HRV by including a randomised controlled trial (RCT) in centrally obese individuals. Figure 1 shows the studies timeline.

Figure 1 - Thesis studies timeline. Recruitment and data collection periods highlighted except for the MARINA study where only data processing is highlighted.



This introduction chapter will provide an insight into the relationship between the ANS and HRV, as the method chosen to assess the cardiac autonomic function, and the relevant dietary aspects, with the aim of recognising literature gaps and to provide background to the design of the studies included in this thesis.

1.1 Cardiovascular disease

Cardiovascular diseases (CVDs) are the leading cause of death worldwide and according to the World Health Organisation report, an estimated 17.5 million people died from CVDs in 2012 which represents 31% of all global deaths. Of these deaths, an estimated 7.4 million were due to coronary heart disease (CHD) (1). Among cardiac deaths, SCD accounts for approximately 50% (2), and most studies suggest the presence of CHD in patients dying of SCD (3). Although mortality from CVD has been declining in the UK, there is a higher number of people living with the disease with rises in treatment and hospital admissions (4). However, SCD rates do not seem to be declining as much as overall CHD mortality (5), representing an area requiring prevention strategies.

1.1.1 Sudden cardiac death

There is no consensus on the definition of SCD. However, according to American College of Cardiology/American Heart Association/European Society of Cardiology Practice Guidelines, SCD is defined as an unexpected death in an apparently healthy subject without an obvious noncardiac cause that occurs within 1 h of witnessed symptom onset or within 24 h of unwitnessed symptom onset and is usually due to a cardiac arrhythmia (3). SCD is the largest contributor to mortality among haemodialysis patients, accounting for one fourth of deaths (6). The pathophysiology of SCD is explained mainly as an electric arrhythmogenic instability and is mostly due to ventricular arrhythmias (2).

1.1.2 Arrhythmia

The heart rhythm is started by automatic electric impulses derived from the sinoatrial node followed by impulse conduction to the ventricles where individual cardiomyocytes

establish coordinated contractions. When the electrical excitation of a certain area of the cardiac tissue is impaired, the heart develops arrhythmias which can lead to SCD (7).

The cardiac action potential is a result of the sequential opening and closing of ion channel proteins in the cardiomyocyte membrane. The conduction of the action potential through the heart is dependent on the electrical coupling between the individual cardiomyocytes, which is mediated by gap junctions. In humans, the cardiac action potential has five different phases. After depolarisation from the sinoatrial node, the membrane potential is brought to the threshold and the voltage-activated sodium (Na^+) channels open, diffusing the Na^+ across the membrane into the cardiomyocyte. The resulting Na^+ current causes further depolarisation of the membrane through a positive feedback loop, which is responsible for the fast upstroke of the action potential. The activation of the fast and slow potassium (K^+) currents result in the early rapid repolarisation. This is followed by a prolonged plateau that results from a balance between the inward currents, formed by the voltage-dependent calcium (Ca^{2+}) channel and Na^+-Ca^{2+} exchanger, and outward currents, formed by the voltage-dependent K^+ channels. The K^+ currents predominate as the Ca^{2+} channels become inactivated, causing further repolarisation and turning the membrane potential into the K^+ equilibrium potential. Finally, after full repolarisation, the membrane potential returns to its resting value (8).

The life threatening ventricular arrhythmias can be initiated by two main mechanisms: abnormalities in impulse initiation (triggered activity and enhanced automaticity) and abnormalities in impulse generation (re-entry). Triggered activity can occur from the premature activation of cardiac tissues by afterdepolarisation, associated with prolonged action potential durations, or through delayed afterdepolarisation, when there is an intracellular Ca^{2+} overload (8). Enhanced automaticity from pacemaker cells (resulting from both voltage- and Ca^{2+}-dependent mechanisms) can increase the action potential discharge rate, resulting in increased heart rate (HR). Re-entry occurs when the electric impulse continues to re-excite the cardiac tissue (9).

Ca^{2+} is the mediator between the electrical activity and the contractile force. When the cardiomyocytes receive the electric impulse, the transmembrane influx of Ca^{2+} via the voltage-

gated Ca^{2+} channels leads to mechanical contraction through interactions of the actin and myosin (10). Abnormal Ca^{2+} dynamics, due to membrane damage causing Ca^{2+} overload, make cardiomyocytes exhibit spontaneous wave-like elevations of Ca^{2+} that propagate within the individual myocytes (Ca^{2+} waves), which induces re-entry and can lead to abnormal excitation in a certain area of the heart (7). Other from the electrophysiological derangements, cardiac arrhythmias can also result from molecular, cellular and/or structural abnormalities in the cardiac tissue, including altered gap-junctional communication, pathological alteration of the cardiac fibroblasts and structural alterations of the myocardium, respectively (7).

Current treatments for arrhythmia include the placement of an implantable cardioverter defibrillator (ICD), which is only accessible to a small number of patients; neural ablation, which has a low long-term success rate; and pharmacological anti-arrhythmic therapy. The arrhythmias mechanisms vary largely and therefore different types of arrhythmias present distinct autonomic triggers with different targets for treatment and prevention (11). This may be the reason for conflicting results in treatment and prevention of arrhythmias, as a pharmacological therapy may be anti-arrhythmic in a patient with heart failure but may have a pro-arrhythmic effect in a patient with myocardial infarction (12). Increasing evidence suggests that the ANS plays a role in the pathophysiology of arrhythmogenesis as a trigger and predisposing factor (13).

1.2 Autonomic nervous system

Clinical evidence has shown the role of the ANS in the development and progression of CVD (14).

1.2.1 Overview

The neural regulation of the cardiac autonomic function is mainly effected through the interplay of the sympathetic and parasympathetic nervous systems (SNS and PNS, respectively). Together, these form the ANS that represents an interface between the central nervous system (CNS) and the body (15). The ANS is responsible for the regulation of the heart's rate, rhythm, and contractility (13), allowing the cardiovascular system to adapt to the various daily life

activities (14). The sympathetic nerves innervate the heart and blood vessels, and the vagal parasympathetic nerves innervate the heart. Sympathetic activity to the heart is regulated by sympathetic nerve fibres that derive from major autonomic ganglia, including superior cervical ganglia, cervicothoracic (stellate) ganglia and thoracic ganglia, that originate from premotor neurons located in the brain stem and hypothalamus, whereas parasympathetic activity to the heart is regulated by the vagus nerve that originates in the nucleus ambiguus in the medulla oblongata (**Figure 2**) (16).

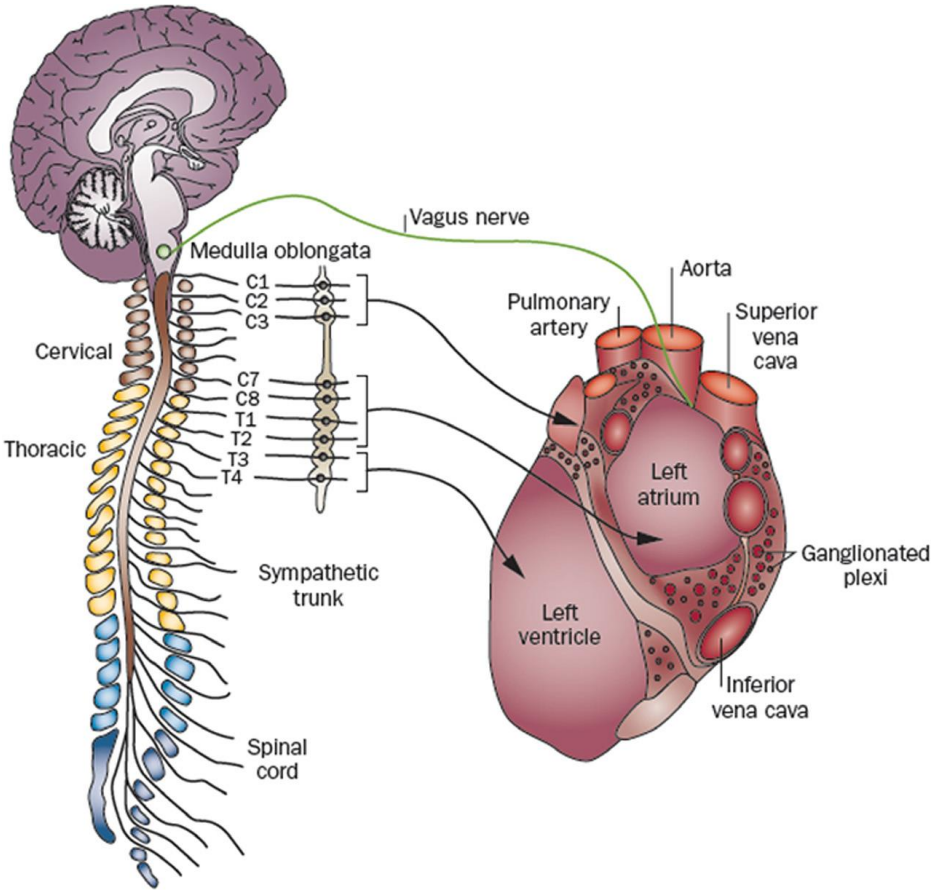


Figure 2 – Cardiac autonomic innervation. Reprinted from (11) with permission from Elsevier. The sympathetic innervation to the heart originates from the superior cervical ganglia, cervicothoracic (stellate) ganglia and thoracic ganglia and the parasympathetic innervation derives from the vagus nerve.

Although automaticity is intrinsic to different cardiac tissues with pacemaker properties, the electrical and contractile activity of the myocardium is largely modulated by the ANS (17). While the effect of vagal stimulation on the cardiac pacemaker cells is to cause hyperpolarisation and to reduce the rate of depolarisation, sympathetic stimulation causes

chronotropic effects by increasing the rate of pacemaker depolarisation. The ANS is, therefore, responsible for the tight regulation of cardiac excitability and contractile function (18).

In healthy individuals, the sinoatrial node is entirely responsible for the regulation of the heart beat and is directly innervated by both vagal and sympathetic efferents. Sympathetic and parasympathetic nerves exert opposite but not symmetrical effects on the heart. The parasympathetic effect on reducing HR is mediated by the synaptic release of acetylcholine (presents a short effect latency and a high turnover rate) which exerts an almost immediate response allowing a beat-to-beat cardiac regulation. The sympathetic effect on raising the HR is mediated by the synaptic release of noradrenaline (presents a longer effect latency and lower turnover rate compared to acetylcholine due to being reabsorbed and metabolised relatively slowly) which exert a slower response and longer lasting effect in the resulting changes in cardiac autonomic regulation compared to the parasympathetic nerves (19). The SNS plays a role in increasing myocardial contractility and peripheral resistance whereas the PNS presents a limited effect in myocardial contractility. Otherwise from the antagonistic influences in the sinoatrial node, the SNS and PNS also modulate the electrophysiological properties of the heart, by influencing ion channel activity.

In humans, the autonomic modulation is mainly regulated by the baroreflexes to control the circulatory homeostasis and this occurs via negative feedback. When the firing rate of the baroreceptors is increased by a rise in systemic arterial blood pressure, the cardiac vagal activity increases whereas the sympathetic activity decreases, resulting in a slower HR, decreased cardiac contractility and decreased peripheral vascular resistance. When the firing rate of the baroreceptors lowers by a decrease in systemic arterial blood pressure, the opposite haemodynamic changes occur (20). A reduced baroreflex control has been associated with increased SCD in patients after myocardial infarction (21).

1.2.2 ANS dysfunction

Cardiac autonomic dysregulation is a hallmark of CVD (22). The sympathetic and parasympathetic neural factors have been studied in a number of cardiovascular (including

hypertension and cardiac arrhythmias), metabolic (including obesity) and renal (including renal failure) diseases, with the attempt to define the role of the ANS as the underlying mechanism in disease as well as investigating potential therapeutic targets (23). The evidence on the sympathetic overactivation and parasympathetic abnormalities in the development of cardiovascular, metabolic and renal disease, including the potential unifying role of inflammation in autonomic dysfunction associated with these diseases, will be summarised below.

1.2.2.1 Hypertension

The pathogenesis of essential hypertension has been widely studied in both animals and humans, with the major mechanisms including the activation of the SNS and renin-angiotensin-aldosterone system (24). Cardiac sympathetic overactivation may contribute to the development, maintenance and progression of hypertension (23). Assessment of sympathetic neural activity by microneurography, measurement of plasma norepinephrine or norepinephrine spillover (based on intravenous infusion of radiolabelled norepinephrine), has shown hyperactivation of sympathetic outflow to the kidneys, heart and skeletal muscle in several established hypertensive states (25,26). In addition, it was shown that the greater norepinephrine levels observed in hypertensive patients is due mostly to an increased release of the neurotransmitter from the adrenergic nerve terminals, as opposed to reduced adrenergic nerve terminals reuptake of the neurotransmitter (25). The mechanisms triggering the sympathetic overactivation remain uncertain and include the following hypotheses: functional impairment of arterial baroreflexes and chemoreceptors that exert inhibitory effects on central sympathetic drive; hyperinsulinaemia causing a pro-sympathoexcitatory effect on the CNS; or stimulation of SNS by circulating humoral factors, including angiotensin II and aldosterone (23).

1.2.2.2 Cardiac arrhythmia

The intrinsic cardiac nervous system receives input from both SNS and PNS and plays a key role in the regulation of the atrial and ventricular function (27). An imbalance of the ANS has a substantial impact on the induction and maintenance of atrial and ventricular arrhythmias (28). **Figure 3** illustrates the arrhythmogenic effects of the SNS and PNS on cardiac

electrophysiology. Increased sympathetic activity in a canine model of SCD preceded immediately the occurrence of ventricular fibrillation and SCD (29).

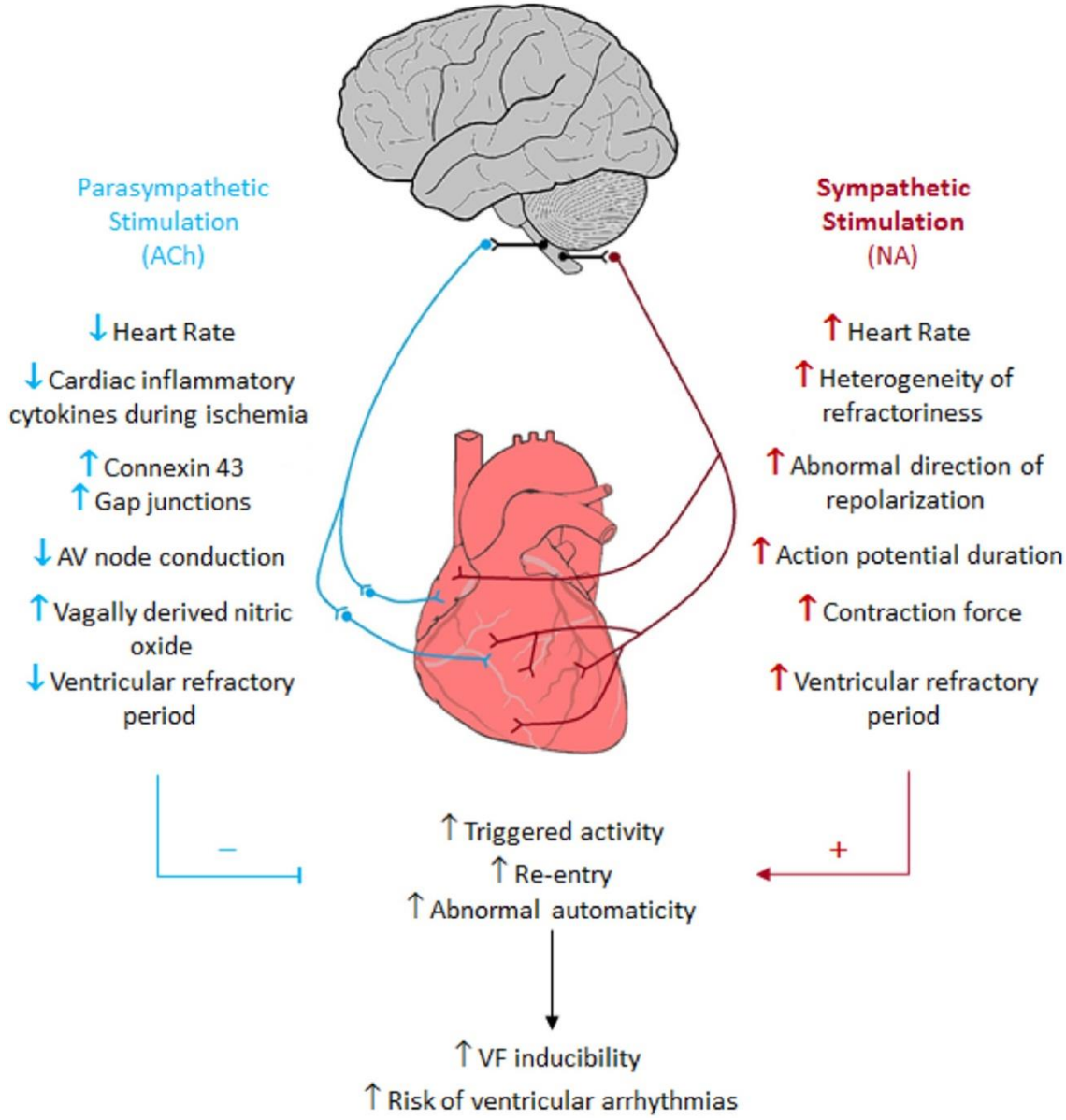


Figure 3 – Arrhythmogenic effects of the sympathetic and parasympathetic stimulation of the heart. Reprinted from (13) with permission from Elsevier. The interplay between PNS and SNS allow the constant adaptation of the cardiovascular system to physiological stimuli. Disruption of the PNS and SNS balance with an enhanced sympathetic stimulation has a significant impact on the initiation of ventricular arrhythmias through increased triggered activity and automaticity or by inducing re-entry. ACh, acetylcholine; NA, norepinephrine; VF, ventricular fibrillation.

1.2.2.3 Overweight/obesity

Overweight and obese individuals are at higher risk of CVD associated with the increased incidence of diabetes, dyslipidaemia, atherosclerosis, renal disease and hypertension (30). In a

previous study, it was shown that obese individuals were more prone to ventricular arrhythmia compared to lean individuals (31). In middle aged adults, obesity is one of the main contributors to an increased risk of SCD (32). Obese adults have raised urinary norepinephrine levels, indicating greater sympathetic outflow, compared to healthy individuals, and centrally obese adults (mostly visceral fat) presented a higher degree of sympathetic activation compared to individuals with subcutaneous obesity (33,34). Alvarez et al. showed a higher sympathetic activity in men with elevated abdominal visceral fat compared to age-, total fat mass- and abdominal subcutaneous fat-matched controls with lower abdominal fat (35).

The underlying cause of the increased sympathetic activity in obesity is not fully understood and it is likely to be multifactorial (36). Nevertheless, many mechanisms have been proposed including, amongst others, inflammation, impaired baroreflex sensitivity, increased non-esterified fatty acids (NEFA) release from excessive visceral adipocytes, hyperinsulinaemia, hyperleptinaemia and the activation of melanocortin 4 receptors (MC4R) in the CNS, which play a role in body weight homeostasis by regulating appetite (37). Obesity has been associated with a chronic low grade inflammation, which may be induced by lipotoxicity through the secretion of pro-inflammatory cytokines by the adipose tissue (38), with a potential contribution to the increased sympathetic activity (the role of inflammation in the ANS is summarised below in section 1.2.3). In individuals with obesity that present with hyperinsulinaemia, the central sympathoexcitatory effects of insulin appear to be blunted leading to a sustained sympathetic activation through the insulin feedback loop (39). The arterial baroreflex activity was also found to be blunted in individuals with central obesity suggesting a causal role in the sympathetic overactivity observed in obesity (40). Leptin is a hormone secreted by adipocytes in direct proportion to adiposity levels and acts in the CNS to decrease food intake by decreasing appetite, and increase energy expenditure by increasing sympathetic activity, predominantly to the kidney and skeletal muscle, with a consequent increase in blood pressure (41). In obesity, leptin resistance has been shown to be selective, with hyperleptinaemia failing to regulate energy balance but maintaining its effects on the

sympathetic outflow and blood pressure, which may explain the tendency for obesity to induce sympathetic overactivity and hypertension (42).

1.2.2.4 Chronic kidney disease

Chronic kidney disease (CKD) is defined by KDIGO (Kidney Disease: Improving Global Outcomes) guidelines as abnormalities of kidney structure or function, that have persisted for at least 3 months, and present health implications (43). CKD severity is classified according to level of albuminuria and glomerular filtration rate (GFR) (**Table 1**), where the category G5 (stage 5) of < 15 ml / min per 1.73 m^2 represents kidney failure, which can be treated only by dialysis or transplantation (43).

Table 1 - Glomerular filtration rate categories in chronic kidney disease.

GFR category	GFR (ml/min/1.73 m^2)	Terms
G1	≥90	Normal or high
G2	60-89	Mildly decreased
G3a	45-59	Mildly to moderately decreased
G3b	30-44	Moderately to severely decreased
G4	15-29	Severely decreased
G5	<15	Kdney failure

GFR, glomerular filtration rate.

Cardiovascular disease is particularly prevalent in CKD (44), and accounts for 43% of all-cause mortality among dialysis patients (45). It has been observed that the risk of SCD is doubled when a patient with CKD stage 5 starts dialysis (46) and in haemodialysis, SCD accounts for two thirds of all cardiac deaths and one fourth of all-cause mortality (45). Most of the CKD patients present with hypertension which plays a key role in the further deterioration of renal function and in the particularly high rate of cardiovascular events. The kidneys present afferent sensory signals and efferent sympathetic innervation, with sympathetic postganglionic fibres innervating all the essential renal structures. Renal sympathetic activity leads to volume retention via Na^+ reabsorption and stimulates renin release with a consequent activation of the

renin-angiotensin-aldosterone system. Angiotensin II can then stimulate sympathetic nerve activity through central mechanisms (47). Sympathetic overactivation is present in CKD patients and it has been demonstrated by the increased concentration of plasma catecholamines as well as greater sensitivity to norepinephrine in these patients (48,49).

In summary, an autonomic dysfunction characterised mostly by a sympathetic overactivity, and to a lesser extent, a reduced parasympathetic activity is associated with increased morbidity and cardiovascular mortality (50,51). Modulation of the ANS through pharmacological or non-pharmacological means improves patients' survival (14). For this reason, it is important to investigate the possible modulatory effects of dietary determinants on autonomic balance on its own or as an adjunct therapy, as this could be a cost-effective and side-effect free way of lowering CVD risk. In addition, as inflammation is a unifying component of CHD, obesity and CKD, this might be an important mechanism whereby the ANS is compromised and may be mediated by diet and / or nutritional status.

1.2.3 Autonomic nervous system and inflammation

The ANS plays an essential role in the physiological regulation of basal conditions as well as in response to acute and chronic stressors. The ANS, primarily the SNS, is activated by stressors, including antigens from foreign substances and tissue damage, and regulates the host defence mechanisms. Immune organs and tissues are innervated by the postganglionic sympathetic neurons and the adrenal medulla is innervated by preganglionic sympathetic neurons, releasing the catecholamines, norepinephrine and epinephrine into the circulation. Therefore, the SNS can have either local or systemic effects on the immune system. On the other hand, the PNS doesn't innervate the primary immune organs (thymus and bone marrow), but provides vagal afferents that transmit immune signals to the brain that regulate sympathetic outflow to immune organs (52). The information on tissue damage or antigen exposure leading to immune activation is conveyed to the CNS by the afferent visceral fibres, including those in the vagus nerves (53).

The acute inflammatory response is protective, allowing for repair of damaged tissue and elimination of invading organisms, and is self-limited leading to complete resolution with return to homeostasis. The acute inflammatory response starts with the migration of the immunocytes (specially neutrophils) to the site of inflammation, guided by chemokines and eicosanoid mediators, including prostaglandins and leukotrienes (54,55). The major phospholipids in human plasma and erythrocytes - phosphocholine and phosphoethanolamine - are also concentrated in macrophage plasma membranes and represent the largest phospholipid stores of dietary *n*-3 and *n*-6 PUFAs in cells and fluids involved in inflammatory responses (56). The fatty acid composition of membrane phospholipids in macrophages and endothelium will influence the inflammatory responses via conversion of the *n*-3 and *n*-6 PUFAs into their respective lipid mediators. The first step in the synthesis of the lipid mediators is the release of the esterified fatty acid – arachidonic acid (AA), linoleic acid (LA), α-linolenic acid (ALA), docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) - from the membrane phospholipids by phospholipase enzymes. These fatty acids can be further metabolised by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) monooxygenases or undergo non-enzymatic oxidation with reactive oxygen species to generate numerous bioactive lipid mediators (57). These bioactive lipid mediators, including the specialised pro-resolving mediators (SPM) derived from the metabolism of LC *n*-3 PUFA, are actively involved in the resolution phase of inflammation, promoting the return to homeostasis (58). The resolution phase stimulates cessation of neutrophils infiltration into affected tissues and promotes the clearance of apoptotic cells, debris and microbes by the macrophages (59). However, when acute inflammation is not resolved or is excessive it can become chronic, leading to tissue damage and contributing to chronic diseases, such as cardiovascular and renal diseases (60). A study demonstrated in mice a novel interaction between pro-resolving lipid mediators and neutrin-1 regulated by the vagus nerve, which stimulates the resolution of the inflammation (61). Neutrin-1 is a neuronal protein that acts as a chemoattractant in axonal migration in peripheral tissues, contributing to local control of leukocyte migration (62).

In the site where an acute inflammatory response occurs, the bioactive oxygenated lipid mediators originating from essential fatty acids are of particular interest due to their nutritional regulation of the response (58).

There is growing evidence of an increased risk of arrhythmia in inflammatory diseases (63,64). A study in patients with stable CHD and a history of myocardial infarction showed that those experiencing ventricular arrhythmia had significantly higher serum high sensitivity C-reactive protein (hs-CRP), a marker of systemic inflammation, compared to those who did not, suggesting that inflammation might play a role in ventricular arrhythmia pathogenesis (65). Inflammation is a key component of the pathophysiology of atherosclerosis, which is the main underlying cause of CVD, and has been suggested to mediate the relationship between autonomic dysfunction and atherosclerosis (66). Briefly, atherosclerosis is an inflammatory disease that involves the endothelium and is characterised by a gradual accumulation of lipids and inflammatory cells within the intima of large arteries (67). It starts with the internalisation of low-density lipoproteins (LDL) in the intima with concomitant endothelial dysfunction and further infiltration and accumulation of LDL in the extracellular matrix; circulating monocytes are then recruited and attached to the vascular endothelium, followed by transmigration into the subendothelial space where they become macrophages; endothelial dysfunction facilitates platelet adhesion and activation (due to disruption of the endothelial-related antithrombotic properties), resulting in the secretion of chemotactic and growth factors, which stimulate migration, accumulation and proliferation of vascular smooth muscle cells and leucocytes in the intima resulting in plaque progression; the LDLs retained in the extracellular matrix become prone to oxidative and enzymatic modifications; oxidised LDLs further perpetuate the pro-inflammatory state through further activation, recruitment and transmigration of monocytes and other inflammatory cells into the intima; the macrophages in the subendothelial space scavenge oxidised LDL, become lipid-rich and convert into foam cells and the accumulation of these lead to the formation of fatty streaks; when macrophages fail to remove accumulated cholesterol from the vessel, they become apoptotic and release cholesterol and pro-thrombotic molecules to the vessel wall, contributing to the plaque formation. As the atherosclerotic plaque progresses,

there is a decrease in vascular smooth muscle cells and formation of immature and leaky new vessels that render the lesions more susceptible to rupture. The plaque rupture exposes the thrombogenic substrates which promote platelet adhesion/activation and aggregation to the vascular surface and the activation of the coagulation cascade. This is followed by thrombus formation which can be clinically manifested as acute myocardial infarction or sudden death (68). Although the causes and risk factors for atherosclerosis are unknown, most cardiovascular risk factors, including high total and LDL cholesterol, low level of high-density lipoprotein (HDL), hypertension, diabetes, obesity, smoking and sedentary behaviour, may increase the risk of developing atherosclerosis and controlling these factors can delay or prevent atherosclerosis (68). In addition, atherosclerosis of the sinus node artery, which is involved in the normal functioning of the sinus node, has been associated with an increased risk of arrhythmia in a retrospective study based on patient's coronary angiograms assessment of coronary artery disease (69). Recently, increased inflammatory activity (increased number of activated T lymphocytes and degeneration of adjacent ganglion cells) was found in the left stellate ganglia from patients with malignant ventricular arrhythmia (70), suggesting that increased inflammation may contribute to ventricular arrhythmia through increased sympathetic activity (71) A study in a canine ischemic model showed that injection of interleukin 1β (IL-1β) into the left stellate ganglia, which stimulated an increase in inflammatory state, increased the susceptibility and promoted ventricular arrhythmia under acute ischemic stress by neuronal remodelling of the left stellate ganglia and consequent increase in sympathetic activity (71).

1.3 Assessment of the autonomic nervous system activity

The autonomic function can be assessed through direct quantification or indirect assessment of sympathetic and/or parasympathetic activity. Direct quantification includes measurements of neurotransmitter levels, by analysing circulating catecholamines (mainly norepinephrine) and other metabolites modulated by the ANS; and sympathetic microneurography, which measures muscle sympathetic nervous activity (72). Indirect assessment includes measurement of HRV, which involves continuously recording HR, and baroreflex sensitivity, which comprises of measures of HR and blood pressure and requires the

intravenous administration of vasoactive drugs (72). Among these different techniques for assessing the ANS activity, HRV is often used for assessing cardiac autonomic function as it is non-invasive, thereby offering potential to be applied in the clinical setting with low patient burden (17). As an indirect method to assess ANS activity, it provides qualitative (and not quantitative) information on the relationship between HRV and cardiac autonomic regulation, i.e., a low or high HRV may reflect a decrease or increase in cardiac autonomic function, but does not quantify the actual cardiac nerve firing rate (73).

1.3.1 Heart rate variability

Heart rate variability is a term that refers to a range of parameters that reflect the variability in the length of the interbeat intervals (IBI), this is, the time intervals between adjacent QRS (Q, R and S being points on the R wave seen on an ECG during ventricular depolarisation, and R being the peak upward deflection) complexes (normal-to-normal (NN) intervals) (74). Relative increases in sympathetic activity cause shorter IBI and relative increases in parasympathetic activity cause the IBI to become longer (75). Overall, HRV is inversely correlated with HR, where a greater HRV is correlated with lower HR, and vice versa (76). However, HR represents only a mean value over time and by doing this it overlooks the irregular behaviour presented by the heart when examined as a beat-to-beat basis. These fluctuations between beats result from the interplay between sympathetic and parasympathetic nervous system and reflect functional capacity of various regulatory systems, including the cardiovascular system, to respond to physiological demands. **Figure 4** illustrates the reciprocal relationship between HR and IBI. As HR increases, IBI and HRV decreases as there is less time between heartbeats for variability to occur. As HR decreases, IBI and HRV increases as there is more time between heartbeats to allow for more variability.

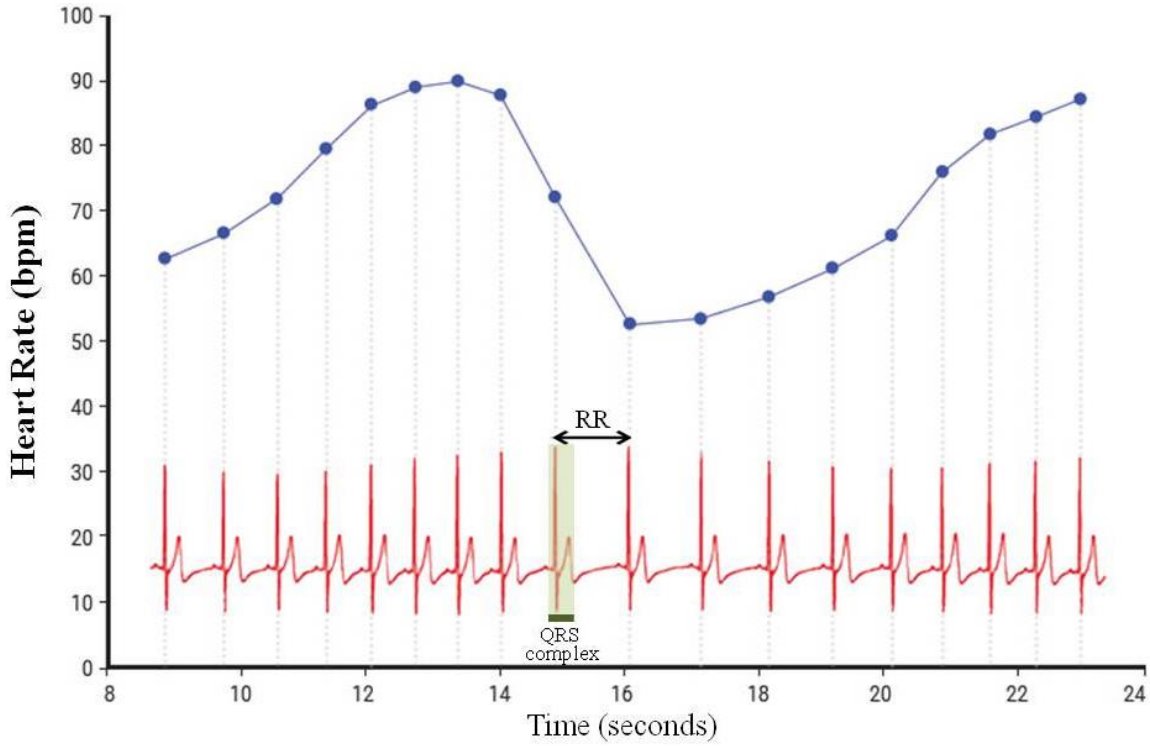


Figure 4 - Example of the relationship between heart rate (dotted blue line) and IBI (RR interval) in the ECG (red line) variations over a short period of time. Adapted from (77).

Low HRV indicates reduced functional regulation of HR by the ANS as it allows less compensatory changes in order to successfully respond to physiological demands, reducing the inherent self-regulatory capacity, adaptability and resilience (77). Low HRV was previously associated with increased risk of SCD and suggested as a powerful predictor of SCD (74). A recent published prospective study, with a median follow up of 13 y. showed that low HRV was associated with an increased risk of SCD in the general population, independent of established cardiovascular risk factors (78). A low HRV has also been shown to be a strong independent predictor of mortality following an acute myocardial infarction (79,80) and risk of cardiac events in the general population (81).

1.3.2 Heart rate variability parameters

Heart rate variability can be quantified using time- and frequency-domain analysis and nonlinear methods. **Table 2** summarises the HRV parameters that are the focus of this thesis. Time-domain analysis is based on the IBIs whereas frequency-domain analysis employ power spectral analysis that provides information about the distribution of power (variance and amplitude) as a function of frequency (time period). The values are expressed as the power spectral density, which is the area under the curve (peak) in a given bandwidth of the frequency

spectrum. A power spectral analysis commonly used is the fast Fourier transformation (FFT), a nonparametric method characterised by discrete peaks for each frequency components. Nonlinear methods also use fractal analysis to study the multivariable, nonlinear and nonperiodicity of HR dynamics. The Poincaré plot is formed of points that correspond to two consecutive IBIs and it explores the non-linear dynamics of HRV in a qualitative or quantitative manner (14). The qualitative method involves visual inspection of the plot pattern, which is usually a fan-like or comet-like shape, and it identifies ectopic beats and technical artefacts (15). Quantitatively, the Poincaré plot can be analysed through geometric procedures, where the transversal axis (SD1) indicates vagal-mediated short-term variability and the longitudinal axis (SD2) reflects overall variability as an inverse function of sympathetic activity (14).

Table 2 – HRV parameters definition. Adapted from (74).

Variable	Definition
Time-domain methods	
SDNN (ms)	Mean of the standard deviations of NN intervals. Reflects the overall and circadian fluctuations in HRV.
SDANN (ms)	Standard deviations of the average NN intervals in all 5 min segments of the recording. Reflects long-term fluctuations in HRV.
RMSSD (ms)	Root mean square of successive differences of NN intervals. Reflects short-term changes in HRV and alterations in parasympathetic respiratory variation.
pNN50 (%)	percentage of adjacent NN intervals that differ by greater than 50 ms. Reflects short-term changes in HRV
Triangular index (Ti)	Number of all NN intervals divided by the maximum of the density distribution. Also referred to as HRV index. Reflects overall HRV.
Frequency-domain methods	
LF (ms^2)	Low-frequency power (0.04-0.15 Hz). Modulated by baroreceptors. Reflects both parasympathetic and sympathetic activity.
HF (ms^2)	High-frequency power (0.15-0.4 Hz). Reflects parasympathetic respiratory modulation.
LF:HF ratio	May reflect relative sympathetic to parasympathetic activity.
VLF (ms^2)	Very low-frequency power (0.003-0.04 Hz). May reflect parasympathetic and renin-aldosterone activity. Represents the majority of total power in HRV.
Non-linear methods	
SD1 (ms)	Standard deviation of the Poincaré plot perpendicular to the line of indentity.

	Refects short-term variability, primarily respiratory sinus arrhythmia.
SD2 (ms)	Standard deviation of the Poincaré plot along the line of indentity. Reflects long-term variability.
Poincaré ratio	the ratio of the SD of beat-to-beat IBI variability (SD1) against the SD of long-term IBI variability (SD2) - Indicates normality of sinoatrial firing patterns.

Since the publication of the Task Force on HRV standards in 1996 (74), there hasn't been an update of the HRV standards guidelines, which is warranted as a vast amount of papers have been published since then describing new HRV methodologies and applications in different contexts.

There are more methods that can be used in HRV analysis that are outside of the scope of this thesis as these have provided more knowledge on the complexity and mathematical characteristics of the variability rather than providing information on the sympathetic and parasympathetic control mechanisms. These include methods to estimate the fractal-like behaviour of HRV, such as the detrended fluctuation analysis (DFA), methods to quantify the entropy rate, such as approximate entropy and sample entropy and methods to measure non-linear dynamical systems and chaotic behaviour (82).

Some considerations need to be made about the use of HRV as the method to assess cardiac autonomic function. Firstly, studies assessing HRV vary in the duration of ECG/IBI recording, devices used, time period measured (day, night, 24 h), report different HRV parameters and vary in the methodology used in the data processing which makes comparisons between studies rather difficult. Secondly, there are disagreements in the literature on the validity of LF power and LF : HF ratio as a measure of sympathetic activity and sympathovagal balance, respectively. Earlier investigations have suggested LF to reflect predominantly sympathetic activity and based on this assumption the LF : HF ratio was proposed as a way to quantify the relationship between sympathetic and parasympathetic activity (73,74,83). However, growing evidence has shown LF to reflect both sympathetic, parasympathetic as well as other unidentified factors and therefore, LF : HF ratio would not reflect an acurate measure of cardiac sympatho-vagal balance (84). More recently, LF power has been suggested as a marker

of baroreflex function, rather than cardiac sympathetic activity (85,86). There is the need to standardise the methodology for analysing and reporting HRV parameters to make it possible to compare results between studies. In addition, future research should focus on the clinical applicability of HRV, such as determining cut-off points for CVD risk as well as assessing the value of HRV for risk predicting in addition to established risk factors, and how dietary determinants can potentially have a role in reducing CVD risk through modulation of HRV.

1.4 Heart rate variability and inflammatory markers

An inverse relationship has been found between HRV and inflammatory markers in both healthy subjects and those with CVD (87). In healthy adults, inverse correlation were found between interleukin-6 (IL-6) and C-reactive protein (CRP) and 10 min frequency-domain HRV parameters (HF and LF) (88) as well as CRP and 24 h RMSSD and pNN50 (75). In healthy adults, the relationship between HRV and IL-6 was also assessed during and after mental stress test but no significant correlations were found (89). However, signal processing difficulties in this study limited the amount of usable HRV data for analysis, which in turn limited the HRV results to a subsample of the cohort. The Cardiovascular Health Study with normoglycaemic adults aged 65 and older with a 15 y follow-up showed an inverse correlation between IL-6 and CRP, and 24 h and day-time SDNN and VLF power (90,91). In newly diagnosed and established diabetic patients, IL-6 presented an inverse correlation with SDNN (92). In patients with moderate to severe CKD, IL-6 was negatively correlated to 24 h SDNN, SDANN and VLF power, and positively correlated with LF power, but these were not statistically significant in the haemodialysis group, where adequacy of dialysis was associated to HRV (93). These results suggest that IL-6 may have an important role in the progression of kidney disease leading up to kidney failure. In patients after acute myocardial infarction, CRP levels were negatively correlated with 24 h SDNN, HF and LF power (94), and in patients with unstable angina pectoris, CRP levels were inversely correlated with SDNN, SDANN, VLF, LF and LF:HF ratio (95). In patients with suspected CHD, hs-CRP was associated with 24 h SDNN, and the association was strongest in those that had a prior myocardial infarction (96). However, in a

study with CHD patients using 5 min HRV analysis no statistically significant correlations were found between CRP and frequency-domain HRV (97).

1.5 Dietary determinants of heart rate variability

In the field of nutritional sciences, the effect of whole diets or dietary components on cardiac autonomic function as a risk factor for risk of mortality from CVD has rarely been considered. Consequently, there is a lack of studies, particularly in humans, assessing the effect of diet and / or nutritional status on HRV. Aspects of diet and nutrition that have been investigated with regards to HRV include energy balance (98–104), although the quality of the evidence base is weak in this field, and LC *n*-3 PUFA (104–112), where there have been a series of RCTs in healthy and patient populations from a group in Aalborg University Hospital, Denmark. Other potential dietary determinants of HRV that have limited amount of evidence published and hence are not the main focus of this PhD, include vitamin D and vitamin B12, where low levels or deficiency has been associated with lower HRV in healthy adults (113) and in children (114), respectively. The energy balance and LC *n*-3 PUFA are the dietary aspects that constitute the main focus in relation to HRV research and their findings are critically reviewed below.

1.5.1 Energy balance

The dynamic balance between the SNS, which is responsible for the mobilisation of energy, and the PNS, which promotes digestion and energy storage, responds to environmental stimuli, such as fasting, in order to adapt the energy metabolism to the body demands (115).

1.5.1.1 Energy restriction in animal models

The cardioprotective mechanism behind energy restriction remains to be elucidated in humans but evidence from animal studies suggest two potential mechanisms that could explain it: decreased mitochondrial production of reactive oxygen species, resulting in reduced oxidative damage to the cardiomyocytes; and increased cellular resilience to stress (116). Energy restriction in rats have shown to increase HRV and decrease oxidative damage to the cardiomyocyte as well as reduce inflammation in the ischemic zone following a myocardial

infarction (98). Oxidative stress plays an important role in ischemic injury to cardiomyocytes, which can lead to depressed myocardial contractility and arrhythmias (117). Rodents maintained on energy restriction have shown reduced amounts of oxidative damage to proteins, lipids and DNA in heart tissue, compared with rodents maintained on an ad libitum diet (118,119). Also, energy restriction have increased the level of heat-shock proteins and other protein chaperones in rodents' heart tissue, showing an activation of cellular stress response pathways (120). Thus, energy restriction could be a potential mediator between reduced inflammation and improved cardiac autonomic function.

1.5.1.2 Weight loss in human studies

Weight loss is achieved through energy restriction by reduction in daily energy intake relative to total energy expenditure in order to promote a negative energy balance. Dietary interventions aiming at weight loss, mostly those that also include healthy lifestyle advice (increase in physical activity), have been shown to be effective in improving markers of cardiovascular disease risk (121–124). Evidence from several studies in obese individuals have reported improvements in HRV, both in time- and frequency-domain as well as short-term and long-term HRV parameters, after diet-induced weight loss (99–104) or bariatric surgery-induced weight loss (101,125–127). Studies on diet-induced weight loss showed increases in time- and frequency-domain HRV parameter using different recording durations (5 min (101), 10 min (104), 3 h (102), 18 h (100) and 24 h (99,103,128)), interventions of different lengths (ranging from 3 to 16 weeks), different study designs with different dietary approaches (very low calorie diet (VLCD), continuous energy restriction, mixed approaches) and with study populations with different degrees of overweight/obesity and different levels of cardiovascular risk (obese but otherwise healthy, with/without diabetes, including/excluding smokers, etc). Bobbioni-Harsh et al. showed statistically significant improvements in all 24 h time-domain HRV parameters after bariatric surgery-induced weight loss in a biphasic pattern with the rapid initial weight loss phase (assessed 3 months after surgery) having the greatest withdrawal in sympathetic activity (increased SDNN and Ti) and enhanced parasympathetic activity (increased RMSSD and pNN50), and the following slow phase of weight loss (assessed 12 months after surgery) having

a partial reactivation of sympathetic activity, shown by a decrease in SDNN and Ti, yet remaining significantly higher than baseline (before surgery) (127). In the same study, the patients after 12 months of surgery were compared to a stable weight control group that was age-, sex- and post-surgery BMI-matched, but no differences in HRV were found between groups. These results indicate that substantial weight loss improved cardiac autonomic function, but it is influenced by the BMI reached after surgery (127). Most of the weight loss studies in an obese sample assessing HRV lack a control group and due to their observational nature preclude the ability to assess a causal link between weight loss and decreased SNS activity / enhanced PNS activity. An RCT in healthy normal weight adults assessing the effects of weight gain followed by weight loss (8 weeks each) in frequency-domain HRV parameters, showed a statistically significant decrease in HF with an increase in LF and LF : HF ratio after weight gain, during both waking hours and sleep-time, that returned to baseline values after weight loss, whereas controls had no change in HRV (129), showing a reversible deleterious effect of weight gain on the autonomic function. However, these results cannot be directly extrapolated to the obese subjects as obesity reflects a long-term chronic weight gain.

1.5.1.3 Fasting and ketone bodies

To maintain a constant body weight, a balance of energy intake is required, alternating between feeding and fasting which trigger an increase or decrease in energy expenditure, respectively, by the action of the SNS. When feeding, sympathetic activity is enhanced resulting in increased HR and food-induced thermogenesis and when fasting, sympathetic activity is supressed resulting in decreased HR and activity (130).

Ketone bodies (acetoacetate, β-hydroxybutyrate and acetone) become an alternative and important source of energy when glucose is in short supply, with their levels increasing during fasting. They are synthesized in the liver from acetylCoA derived mainly from fatty acid oxidation and then transported to extrahepatic tissues, including the brain, heart and skeletal muscle, through the blood stream (131). A study in rodents reported β-hydroxybutyrate to inhibit the SNS via antagonist effects on the G protein-coupled receptor 41 (GPR41), also known as free fatty acid receptor 3 (FFA3), which is abundant in the sympathetic ganglia in

mice and humans (132). However, another studied found the opposite, reporting β-hydroxybutyrate as a FFA3 agonist, and FFA3 expression and function was found to be unevenly distributed in the rat sympathetic ganglia (133). Whereas Kimura et al. (132) provided in vivo, cellular and biochemical evidence of β-hydroxybutyrate as an antagonist of FFA3, the experiments by Won et al (133) revealed agonist activity. It cannot be ruled out the fact that β-hydroxybutyrate may act as a partial agonist depending on the context, such as drug concentrations and receptor saturation, and thus may produce different effects (133). In addition, the studies presented different experimental conditions, such as using rats vs. mice and different ages of the animals. Due to the mixed evidence, further studies are warranted to resolve these opposing results, as the former study could lead to a reduction in SNS activity but the latter in increased SNS activity. This would potentially translate into either improved or increased risk of CVD respectively. Although these studies present opposite findings on a potential link between β-hydroxybutyrate and SNS activity, evidence of this mechanism hasn't been studied in humans. In addition, caution should be considered as rat or other animal studies do not always translate to human studies. Methodological issues arising from animal studies include the use of different animal species and strains, which present diverse metabolic pathways and drug metabolites thus resulting in variation in efficacy; the lack of standards in blinding and randomisation methods used; and the challenge to accurately determine the equivalent human dosages of investigational components given in animals due to different blood and body mass volumes, amongst others (134,135). Therefore, if β-hydroxybutyrate acts as a FFA3 antagonist as reported in the first study, this could be a potential mechanism whereby intermittent fasting might be effective in reducing SNS activity, hence lowering the risk of SCD.

1.5.1.4 Physical Activity

A meta-analysis of 12 and 13 studies showed a significant effect of aerobic exercise training interventions (at least 4 week duration) on IBI and HF (136), respectively. A prospective study assessing the effect of physical activity in normal weight, overweight and obese middle-aged and elderly subjects showed that regardless of weight, 24 h SDNN had the

greatest increase in subjects exercising regularly (≥ 2 h per week) (137). In addition, obese subjects exercising regularly presented higher 24 h HF and LF power and lower LF : HF ratio compared to sedentary obese subjects, even after adjusting for sex, age, diabetes, hypertension, use of β-blockers and smoking status (137). A small study in an elderly sample assessed the effect of a 14-week intervention with interval training on HR/IBI and time- and frequency-domain HRV parameters over 24 h, day- and night-time, showing an increase in parasympathetic activity with the following results: significant increase in 24 h and day-time IBI with the respective decrease in HR; RMSSD and pNN50 significantly increased in the night-time and RMSSD also increased over 24 h and day-time; and HF and LF power significantly increased in the night-time while LF : HF ratio significantly decreased in the day-time (138).

1.5.2 Long chain *n*-3 polyunsaturated fatty acids

There is some evidence suggesting an antiarrhythmic role of LC *n*-3 PUFA which is thought to be related to the reduction in cardiovascular mortality/SCD observed in some (139–148) (but not all (149–155)) of the supplementation/dietary intervention studies.

1.5.2.1 Metabolism and sources

The major marine or LC *n*-3 PUFA are eicosapentaenoic acid (20:5*n*-3, EPA) and docosahexaenoic acid (22:6*n*-3, DHA), which can be endogenously derived from the downstream metabolism of α-linolenic acid (18:3*n*-3, ALA), an essential fatty acid. ALA can be converted to EPA and DHA through the action of desaturation and elongation enzymes, but the rate of this conversion is restricted to a narrow range in adults (156). . In studies using stable isotope tracers, the estimated conversion of ALA to EPA was up to 8% in men compared to 21% in women and the conversion from ALA to DHA was shown to vary from undetectable amounts up to 4% in men and up to 9% in women (157). Consequently, these LC *n*-3 PUFA are mostly obtained preformed from the diet (158). **Figure 5** shows the metabolic interconversion of *n*-6 and *n*-3 PUFA.

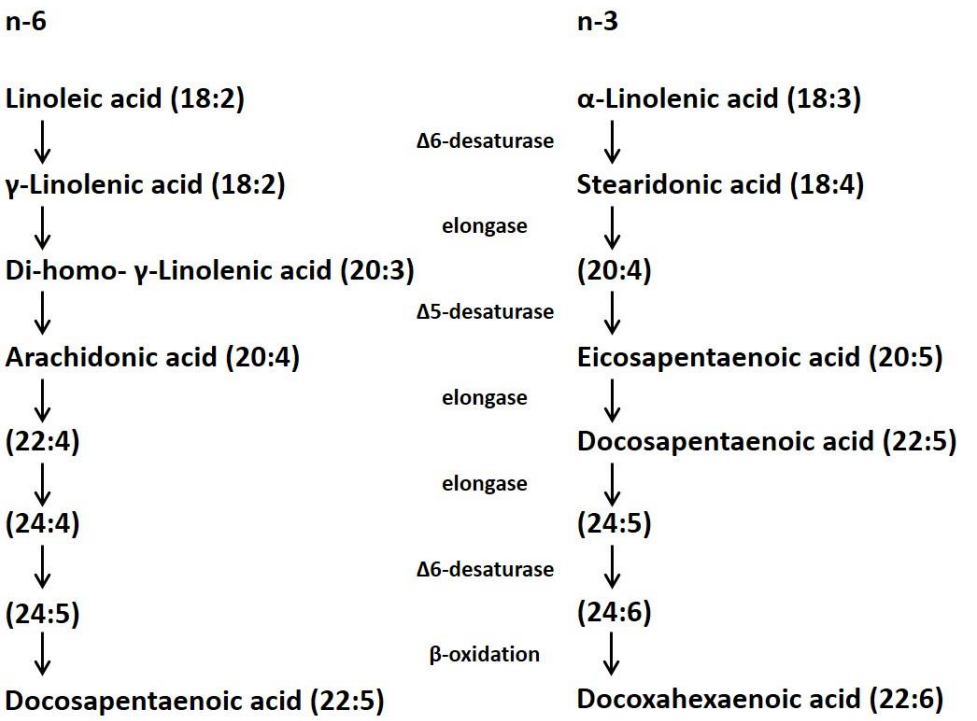


Figure 5 – Metabolic interconversion of n-6 and n-3 PUFA. Adapted from (157).

The main dietary source of EPA and DHA is seafood, but they are also found in red meat, organ meat (liver and brain), in eggs and, in very small amounts, in dairy products (159). SACN have made recommendations for a minimum level of fish or LC *n*-3 PUFA consumption for the general population which includes a minimum of two portions of fish per week, one of which should be oily fish (160). One portion of white (≈ 150 g, cooked) and one portion of oily fish (≈ 80 g, cooked) per week provide approximately 0.45 g LC *n*-3 PUFA per day. The food sources of LC *n*-3 PUFA are presented in **Table 3**. Oily fish, such as salmon, herring, anchovies and mackerel are the richest sources of EPA + DHA. The recommendations for EPA and DHA intake vary worldwide and are compiled in Appendix 1.

Table 3 – Food sources of LC *n*-3 PUFA. Reprinted from (144) with permission from Elsevier.

Common Dietary Sources	EPA, mg/100 g	DPA, mg/100 g	DHA, mg/100 g	EPA+DHA, mg/100 g
Anchovy	763	41	1,292	2,055
Herring, Atlantic	909	71	1,105	2,014
Salmon, farmed	862	393	1,104	1,966
Salmon, wild	411	368	1,429	1,840
Mackerel, Atlantic	504	106	699	1,203
Bluefish	323	79	665	988
Sardines, Atlantic	473	0	509	982
Trout	259	235	677	936
Golden bass (tilefish)	172	143	733	905
Swordfish	127	168	772	899
Tuna, white (albacore)	233	18	629	862
Mussels	276	44	506	782
Striped bass	169	0	585	754
Shark	258	89	431	689
Pollock, Atlantic	91	28	451	542
Oysters, wild	274	16	210	484
King Mackerel	174	22	227	401
Tuna, light (skipjack)	91	17	237	328
Snapper	48	22	273	321
Flounder and sole	168	34	132	300
Clams	138	104	146	284
Grouper	35	17	213	248
Halibut	80	20	155	235
Lobster	117	6	78	195
Scallops	72	5	104	176
Blue Crab	101	9	67	168
Cod, Pacific	42	5	118	160
Shrimp	50	5	52	102
Catfish, farmed	20	18	69	89

1.5.2.2 Cardioprotective effects

Many prospective observational studies and large secondary prevention RCT after myocardial infarction such as the DART study and GISSI-Prevenzione (139,140) have assessed the effects of fish or LC *n*-3 PUFA consumption on CVD outcomes. The GISSI-Prevenzione trial showed that treatment with LC *n*-3 PUFA supplements significantly reduced SCD within 4 months after a myocardial infarction (139), and the reduction of life-threatening ventricular arrhythmias was suggested as the mechanism underlying such benefit. It is important to point out that over 90% of the patients enrolled in the GISSI-Prevenzione trial were concomitantly taking aspirin which its therapeutic actions are exerted through acetylation of COX-2, which in turn is involved in the synthesis of SPMs from EPA and DHA, namely E-series resolvins and D-

series resolvins, respectively. This could have potentiated the anti-inflammatory effects of LC *n*-3 PUFA which could have been the mechanism by which these cardioprotective effects were observed, however a recent study reported that the addition of aspirin to LC *n*-3 PUFA supplementation did not significantly change the extent by which LC *n*-3 PUFA supplementation alone increased precursors of E- and D-series resolvins as well as Resolvin E1, E2 and E3 (161). GISSI Heart Failure study did not show a reduction in SCD but it showed a reduction in all-cause mortality and hospital admissions attributed presumably to an arrhythmic cause (141). The DART study, a secondary prevention RCT in men following a myocardial infarction which studied the effect on total mortality of three dietary factors in eight different combinations (reduction of fat intake to 30% of total energy and increase of polyunsaturated/saturated ratio to 1.0; at least two portions of oily fish per week; and increased fibre intake to 18 g per day), including a group with no dietary advice, reported significantly reduced mortality in the patients randomly assigned to oily fish intake compared to those not given fish advice (relative risk [95% CI], 0.71 (0.54-0.93]) (140). From several meta-analyses performed, the overall findings indicate that consumption of fish or fish oil significantly reduced the risk of CHD mortality, including fatal myocardial infarction and SCD, in populations with and without established CVD (142–146). Furthermore, higher doses of LC *n*-3 PUFA and longer duration of intervention were found to be protective against non-fatal CHD events, as reported in the JELIS trial which supplemented with purified EPA (162). These studies provide strong evidence of the cardiovascular benefits of LC *n*-3 PUFA consumption. A systematic review performed by Wang et al. concluded that increased consumption of LC *n*-3 PUFA from fish or fish oil supplements reduced rates of all-cause mortality, cardiac and sudden death (147). Another systematic review by Delgado-Lista et al. reported that LC *n*-3 PUFA, when administered as food or in supplements for at least 6 months, reduces cardiovascular events by 10% (OR [95%CI]; 0.90 [0.85, 0.96]), cardiac death by 9% (OR [95%CI]; 0.91 [0.83, 0.99]) and coronary events by 18% (OR [95%CI]; 0.82 [0.75, 0.90]), while showing a trend for a lower total mortality (148). The authors concluded that LC *n*-3 PUFA are effective in cardiovascular and coronary events and cardiac death prevention, mainly in those with high cardiovascular risk

(148). A meta-analysis of 19 prospective studies that measured fatty acid composition, EPA and DHA were associated with lower risk of CHD (163).

Although several epidemiological, observational and interventional studies provided evidence on the reduced risk of cardiovascular mortality and SCD with a high intake of LC *n*-3 PUFA, especially in patients with previous myocardial infarction, there are other studies reporting contradictory results. The DART-2 study, a RCT in men with angina, reported a higher risk of cardiac and sudden death in those advised to eat oily fish, particularly those provided with fish oil capsules (149). The OMEGA multicentre study did not report any significant reduction in SCD or coronary events in patients that were supplemented with LC *n*-3 PUFA in addition to the current guideline-adjusted therapy care, including the use of β-blockers, statins, angiotensin-converting enzyme inhibitors and revascularisation procedures. The results from this study add the possibility that LC *n*-3 PUFA might not provide additional benefits when patients are receiving already optimum conventional medical therapy (150). In addition, the authors of the OMEGA study acknowledged that the statistical power of 80% had not been reached (but instead a 44% statistical power) due to both an overestimation of the effect of LC *n*-3 PUFA and a lower than expected event rates. The more recent fish oil supplementation studies, including the Alpha Omega (EPA and DHA enriched margarine), ORIGIN, SU.FOL.OM3 and the RISK & PREVENTION trials did not show a reduction in major cardiovascular events in the intervention group (151–154). However, some considerations should be highlighted such as: the Alpha Omega comparison group used for statistical analyses not being a true placebo but a combination of placebo and ALA-containing margarines, and the fact that the dose of EPA and DHA provided being smaller compared to the earlier trials; SU.FOL.OM3 had an interval between the initial coronary event and randomisation of the participants into the study group of a median of 101 days; the ORIGIN patients the use of concomitant therapies may have prevented an effect from low dose of LC *n*-3 PUFA supplementation; RISK & PREVENTION trial was also acknowledges by the authors to not have reached statistical power sue to lower rates of the cardiovascular events (164). A meta-analysis by Rizos et al. reported that overall LC *n*-3 PUFA supplementation was not associated

with reduced risk of cardiac or sudden death, rejecting the antiarrhythmic mediated LC *n*-3 PUFA effect (155). Another systematic review and meta-analysis in adults with peripheral arterial disease reported no protective associassion between *n*-3 PUFA supplementation and clinical cardiovascular outcomes, including cardiovascular death (165). A recent evaluation of the quality of evidence on LC *n*-3 PUFAs and CVD concluded that there is a high strength of evidence of no effect of LC *n*-3 PUFA supplementation on risk of major adverse cardiovascular events, all-cause death and SCD, and low strength of evidence of no effect of LC *n*-3 PUFA supplementation on risk of CVD death, CHD death, total CHD and myocardial infarction, amongst others (166). Nevertheless, accumulated evidence has been underpowered to detect small underlying effects because these events are relatively rare and not always classified correctly, and thus need very big sample size to reach statistical power. The lack of protective effect from fish oil supplementation in the more recent trials in contrast to the beneficial effects of the earlier trials of fish oil supplementation and epidemiological studies, has led the more recent guidelines in management of patients with stable ischaemic heart disease to not include a recommendation for *n*-3 PUFA supplementation (167), and has been a focus of debate (168,169). The difference in outcome between the trials could be attributed to different LC *n*-3 PUFA tissue levels at baseline, differences in the type and dosage of fish oils, differences in standard medical care that might be masking a potential benefit from LC *n*-3 PUFA (such as use of statins) and whether the trial design was for secondary or primary prevention. Bioavailability issues could be another reason for the lack of effect of fish oil supplementation as it has been shown that different chemical forms of EPA+DHA supplements have different bioavailability, with EPA+DHA bound to phospholipids likely to have the greatest bioavailability, and EPA+DHA bound to recombined triglycerides having greater bioavailability than natural triglycerides, and finally the least bioavailability when bound to ethyl esters (170). Moreover, the most common EPA+DHA supplements used in the intervention trials - ethyl esters - have been shown to have higher bioavailability when consumed with a high fat meal compared to a low fat meal (171). Since the more recent trials have advised the subjects to take their supplements with breakfast, which in many of the study population countries consists of a low-fat meal, the poor bioavailability could have contributed to the neutral effects observed (168).

However, the lack benefit reported in the more recent trials does not necessarily mean that LC *n*-3 PUFA are ineffective in cardiovascular protection, but mean that they were not effective in the context in which they were studied (172). At the moment there are three ongoing CVD prevention studies aiming at providing further evidence on the effects of LC *n*-3 PUFA supplementation: ASCEND, a primary prevention RCT of 1 g/day LC n-3 PUFA (EPA and DHA as ethyl esters) and/or aspirin in 15,480 patients with diabetes and a combined primary outcome of serious vascular events (ClinicalTrials.gov Identifier NCT00135226); REDUCE-IT, a parallel arm, double blind RCT comparing 4 g/day of Vascepa (EPA ethyl ester) with statin therapy vs. statin alone in 8,000 reducing cardiovascular events in patients with hypertriglyceridaemia, established CVD or at high risk of CVD (ClinicalTrials.gov Identifier NCT01492361); and STRENGTH, a parallel arm, double blind RCT comparing 4 g/day Epanova (*n*-3 carboxylic acids) plus statins vs. corn oil placebo vs. statins in 13,000 adults with high CVD risk on major coronary events (ClinicalTrials.gov Identifier NCT02104817).

1.5.2.3 Antiarrhythmic effects

It has been shown that LC *n*-3 PUFA are preferentially incorporated in cardiomyocytes (173) and cardiac tissue EPA and DHA was highly correlated with erythrocyte EPA and DHA in humans (174). There are direct effects of LC *n*-3 PUFA on the generation and duration of the cardiac action potential that stabilise the cardiomyocyte membranes in studies on cell cultures (175). By inducing direct conformational changes of the cell membrane, the EPA- and DHA-enriched cardiomyocytes have an effect on the ion channel function, as well as on other membrane-bound proteins. Consequently, there is an increase in the depolarising stimuli needed to induce an action potential, resulting in reduced automaticity. In addition, the recovery from the inactive state is promoted, with an increase in the refractory period. These two effects combined make the myocardium less excitable, hence less prone to an arrhythmic event (176). Another indirect effect of the LC *n*-3 PUFA on the cardiomyocyte membrane is the inhibition of the synthesis of AA which may result in altered production of eicosanoids. The alteration in the type and amount of eicosanoids generated, which in turn will originate different lipid mediator profiles, has been suggested as an explanation for the anti-arrhythmic properties of LC *n*-3

PUFA via the effects of these changes on intercellular Ca^{2+} release and Ca^{2+} channels (173). Another less direct mechanism proposed include an antiarrhythmic effect of LC *n*-3 PUFA mediated by a reduced agonist affinity for β-adrenergic receptors, observed in cultured rat cardiomyocytes exposed to varied arrhythmogenic agents in the absence of confounders such as neurotransmitters or hormones (177). This could be a potential mechanism whereby LC *n*-3 PUFA could improve the cardiac sympathetic-vagal balance.

The potential antiarrhythmic effect of LC *n*-3 PUFA has been tested in patients with an automatic implantable cardioverter defibrillator (small battery-operated pacing device implantable under the skin to help control life-threatening arrhythmias by delivering an electrical impulse when abnormal rhythms are detected) but the results reported were inconsistent, where Leaf et al. (178) suggested a beneficial antiarrhythmic effect but both Raitt *et al* and Brouwer *et al* found no effect of fish oil supplementation in reducing risk of ventricular arrhythmia (179,180). A meta-analysis by Brouwer et al. that included a combined analysis of three large RCTs in the USA and Europe did not support a protective effect of LC *n*-3 PUFA from fish oil against ventricular arrhythmias in patients with an implantable cardioverter defibrillator (181). However, the data suggested that the effect from fish oil might be dependent on the underlying disease and consequent cardiac electrophysiological condition. This might explain the beneficial effect of fish oil in reducing death rates in the GISSI-Prevenzione trial of patients with a recent myocardial infarction (139) but, on contrary, the increased death rates in the DART 2 trial of patients with stable angina (149). A systematic review by Leon et al. that included 12 RCTs showed no beneficial effect of fish oil supplementation on arrhythmic events or SCD but found a significant reduction in cardiac deaths, mostly related to a reduction in deaths from coronary events (182). However, 92% of the patients included in this systematic review on the effects of fish oil in SCD derived from two large trials (GISSI-Prevenzione and JELIS) and there was significant heterogeneity in the implantable cardiac defibrillator trials assessing arrhythmic events, which could have affected the validity of the analysis. Although the Alpha Omega trial did not find an effect of fish oils on

major cardiovascular events, a secondary analysis of the data showed a reduction in ventricular arrhythmias in the fish oil group (183).

The contradictory results on the antiarrhythmic effect of LC *n*-3 PUFA found in human studies could be due to differences in the arrhythmogenic mechanisms underlying the different type of populations included in the studies (184). Although having the antiarrhythmic properties described above, LC *n*-3 PUFA are capable of promoting re-entry in a susceptible cardiac tissue substrate (i.e. when pathological conditions trigger electrical and fibrotic remodelling of the myocardium), facilitating re-entrant arrhythmias (185). For this reason, the risk of having life-threatening ventricular arrhythmias can be either lowered or raised depending on the mechanism of arrhythmia initiation. Thus, when triggered activity presents as the predominant mechanism of arrhythmia initiation (such as after a myocardial infarction), the LC *n*-3 PUFA would have a beneficial effect in reducing arrhythmic events, whereas when re-entry presents as the main mechanism of arrhythmia initiation (such as in patients with ischaemic heart disease with no history of myocardial infarction), LC *n*-3 PUFA could induce arrhythmic events (184). This finding is important because the advice given towards an increased intake of LC *n*-3 PUFA supplements or oily fish should be tailored to individual patients considering the arrhythmogenic mechanisms associated with the underlying disease (185).

1.5.2.4 Long chain *n*-3 PUFA and heart rate variability

It has been proposed that HRV is influenced by LC *n*-3 PUFA. **Table 4** shows compiled evidence from supplementation studies testing the effect of LC *n*-3 PUFA on HRV. Results from supplementation studies are inconsistent with some studies showing a positive effect (105,109,186), others showing an effect only in men (107) and others showing no effect (110,111). A 12 week RCT fish oil supplementation study has found a strong positive association between DHA in granulocytes and IBI, SDNN, SDANN and pNN50 measured over 24 h Holter monitoring at baseline before supplementation, and increased HRV parameters after 6.6 g LC n-3 PUFA supplementation only in men with low baseline HRV (SDNN below median – 150 ms), but not in women (107). Studies report different durations of HRV recordings which makes it more difficult to compare between studies since it has been shown to

affect HRV measures, mainly time-domain parameters such as SDNN, Ti and SDANN (74). Christensen group has run an RCT in 140 dialysis patients assessing the effect of 2 g fish oil vs. olive oil in 24 h SDNN but to date, the results haven't been published. A study by our group reported that the longer-phase HRV parameters, VLF power and SDANN, were increased during sleep-time following 1 year LC *n*-3 PUFA supplementation in middle-aged healthy subjects (186), underlying the importance of assessing the relationship between tissue LC *n*-3 PUFA and HRV parameters.

Table 4 – Effect of LC *n*-3 PUFA on HRV parameters.

Population studied	Type of study, length and daily dose of LC n-3 PUFA vs. control oil	HRV parameters	LC *n*-3 PUFA effect on HRV parameter
MI survivors (105) **(n = 49)**	RCT, 12 weeks, 4.3 g EPA + DHA vs. olive oil	24 h SDNN	↑
Haemodialysis patients (106) **(n=29)**	Pilot RCT, 12 weeks, 5.2 g n-3 PUFA vs. olive oil	SDNN	positive correlation between n-3 PUFA content in cell membranes and HRV
Healthy subjects (107) **(n = 60)**	RCT, 12 weeks, 3g EPA + 2.9g DHA vs. 0.9g EPA + 0.8g DHA vs. olive oil	SDNN pNN50 RMMSD	↑ (M) ↑ (M) ↑ (M) dose-dependent effect of n-3 PUFA on HRV (M)
MI patients (108) **(n = 10)**	Cross-over trial, 4 weeks, 3g or 6 g n-3 PUFA	24 h LF, HF & LF : HF ratio	↓ LF : HF ratio
CHD patients with previous Myocardial infarction (109) **(n = 18)**	RCT, 2 x 4 months, 225mg EPA + 585mg DHA vs. 50:50 mix corn & olive oil	HF	↑
Stable post myocardial infarction patients (110) **(n = 38)**	RCT, 12 weeks, 460mg EPA + 380mg DHA vs. usual care	Time and frequency domain	No effect
Haemodialysis patients (111) **(n = 30)**	RCT, 12 weeks, 920mg EPA + 760mg DHA vs. olive oil	Time domain	No effect
Obese subjects (104) **(n = 67)**	RCT, 12 weeks, 0 g vs. 0.52 g vs. 1.04 g vs. 1.56 g DHA-rich fish oil	Resting 20 min frequency-domain HRV	↓ LF : HF Dose-dependent effect
Healthy with moderate hypertriglyceridaemia (112) **(n = 26)**	RCT, 8 weeks, 0.85 g vs. 3.4 g EPA + DHA vs corn oil	RMSSD HF	↑ after 3.4 g EPA + DHA Trend after 3.4 g EPA + DHA

(M) effect seen in Men only.

Finding dietary determinants that may have a positive impact on HRV may be a side-effect free, cost effective and sustainable way of lowering the risk of CVD in different types of populations.

1.6 Research question

The primary research question of this PhD is: "Can diet and/or nutritional status modulate HRV and therefore reduce the risk of SCD?". Specific questions include: "Are there dietary determinants of HRV in healthy, centrally obese and chronic kidney disease patients?", and "What are the mechanisms that mediate the potential effects of diet on HRV?".

1.6.1 Aims and hypothesis

The overarching hypothesis of this research is that diet may be a determinant of cardiac autonomic function, more specifically it is hypothesised that the LC *n*-3 PUFA status will be positively associated with HRV and that intermittent energy restriction will lead to greater increases in HRV when compared to continuous energy restriction. Thus, the aim of the doctoral research programme was to investigate relationships between HRV and aspects of diet that may influence sympathetic and parasympathetic activity.

Specific aim 1: investigate the relationship between tissue LC *n*-3 PUFA status and HRV in healthy and diseased populations.

Specific aim 2: compare the effects of 2 weight loss strategies - intermittent fasting (weekly 48 h very low energy diets) and continuous energy restriction (daily moderately low energy diets) – on HRV, and to investigate mechanisms that may mediate any differential effects observed.

Objectives were met by running the following studies:

1) OM3GA study – Observational pilot study in a healthy population comparing vegans with age/sex/BMI-matched omnivore controls, representing populations with low and high tissue LC *n*-3 PUFA status, respectively.

   Hypothesis: vegans have lower HRV and shorter IBI compared to omnivores.

2) FISHH study – Feasibility cross-sectional study in chronic kidney disease patients that recently started haemodialysis to characterise the variability of LC *n*-3 PUFA status and establish a potential relationship between LC *n*-3 PUFA and HRV.

   Hypothesis: a biomarker of longer-term LC n-3 PUFA intake (EPA+DHA as a % of total fatty acid content of erythrocyte membranes) will be independently positively associated with HRV in patients with Stage 5 CKD.

3) Met-IER study – RCT comparing the effects of intermittent and continuous energy restriction diets on HRV in centrally obese, and otherwise healthy individuals.

   Hypothesis: centrally obese adults will have greater increases in HRV following weight loss achieved by intermittent energy restriction (modified "5:2 diet") compared to equivalent weight loss achieved by continuous energy restriction.

Chapter 2 Methods

2.1 Heart Rate Variability analysis

2.1.1. Heart Rate Variability devices

Heart rate variability was measured using the Actiheart monitoring equipment (CamNtech Ltd, Cambridge, UK) for the MARINA study (conducted prior to this PhD 2007-2011), the reproducibility study, and the OM3GA and FISHH cross-sectional studies (conducted as part of this PhD programme between 2012 and 2015). Actiheart monitoring was the least invasive way at the time to measure inter-beat intervals for the analysis of HRV, the alternative being the Holter monitor, an ambulatory electrocardiogram (ECG) device which is about the size of a pack of cards (weighing up to 500 g) and therefore is worn on a belt around the waist. The final study of this PhD research (the Met-IER study, 2016) measured HRV using the eMotion Faros Sensor 180° device (Mega electronics Ltd, Finland), which became available just before the start of this last doctoral study and hence was used instead of the Actiheart monitors. The eFaros monitors not only were able to record ECG raw data but also presented a superior quality in recording the IBIs (with less noise and better signal) and the Cardiscope software for HRV analysis also revealed superior quality compared to the Actiheart software, mainly due to the decrease in the task subjectiveness.

The Actiheart is a small, light-weight (<10 g) waterproof device with two sensors that records acceleration (counts per minute, cpm) as a measure of physical activity, HR, and all IBI, that is, the time-intervals between 'R' or ′normal′ spikes of the QRS (Q, R and S being points on the R wave seen on an ECG during ventricular depolarisation, and R being the peak upward deflection) complex, for approximately 24 h. Studies have shown Actiheart to be technically reliable and valid as a monitor of HR (187), producing values of HRV not significantly different from those produced from the Holter monitor (188).

The eMotion Faros 180° is a small, portable, light-weight (13 g) and wireless 2-lead ECG recorder and transmitter that records ECG data internally (offline mode) or communicates

remotely via Bluetooth (real-time). It also records breathing and it has an in-built 3-axis accelerometer that records acceleration (cpm) as a measure of physical activity.

2.1.2. Fitting the Actiheart and eMotion Faros devices

Before fitting any of the devices, adequate skin preparation is needed in order to obtain a good quality recording, where the ECG signal captured is strong while having an overall low noise level. The skin preparation involved shaving of chest hair when needed, use of alcohol wipes to clean and dry the skin and use of an abrasive pad (Unilect^{TM}) to remove the top layer of skin cells. Two ECG electodes (SP-50, 50 mm round, Pulse Medical) were placed on the chest to fit the monitors.

For the Actiheart monitor, the first sensor was attached to an electrode and placed in the centre of the chest around the fourth intercostal space where the round end of the Actiheart was clicked to it. The second sensor was attached to another electrode and the wire was used to position the sensor as shown in **Figure 6**. The accelerometer component is located in the first sensor, and the Actiheart needs to be placed with the cable as horizontal as possible in order to obtain an accurate measurement of activity. A 10 degree tilt, as shown in **Figure 7**, would introduce 3% activity reading error.

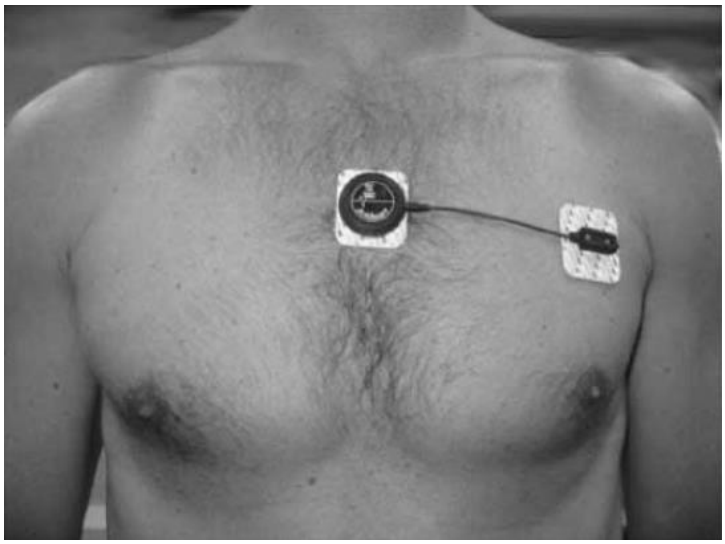


Figure 6 – Actiheart position in the chest. Reprinted rom (187) with permission from Elsevier.

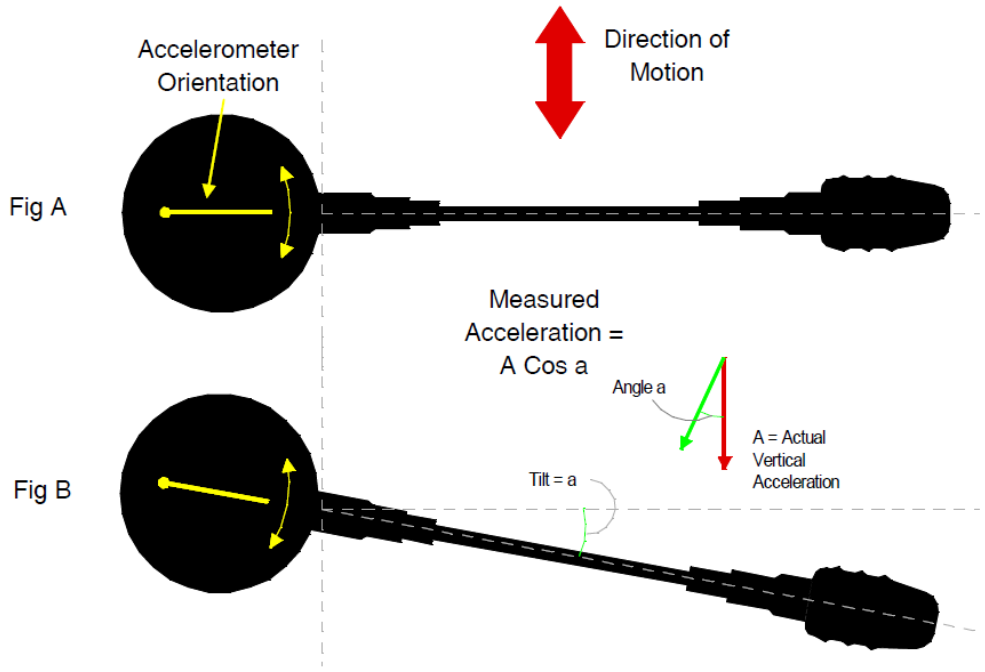


Figure 7 – Effect of tilting the monitor on the accelerometer orientation and consequent introduction of activity reading error.[1]

The electrodes for the eMotion Faros monitor were placed along the electrical axis of the heart as shown in **Figure 8**. The placement of the electrodes followed the locations of electrodes right arm (RA) and left leg (LL) in the Mason-Likar modification of the standard 12 lead ECG (189): the negative electrode was placed in the right infraclavicular fossa (below the right clavicle), and the positive electrode on the left anterior axillary line, halfway between the costal margin and the crest of the ilium. The electrodes would be clipped to the cable endings of the monitor before attaching them to the chest.

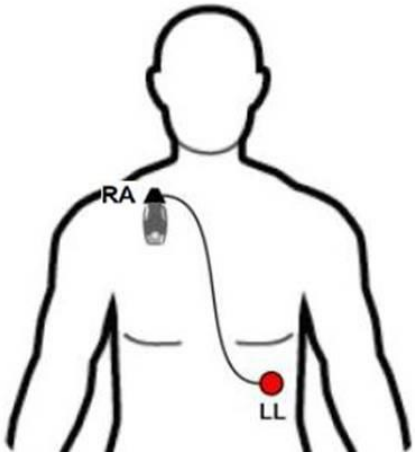


Figure 8 – eMotion Faros position in the chest.

[1] From the Actiheart guide to getting started 4.0.37 (April 2010)

2.1.3. Recording of the IBI and HRV analysis using the Actiheart device

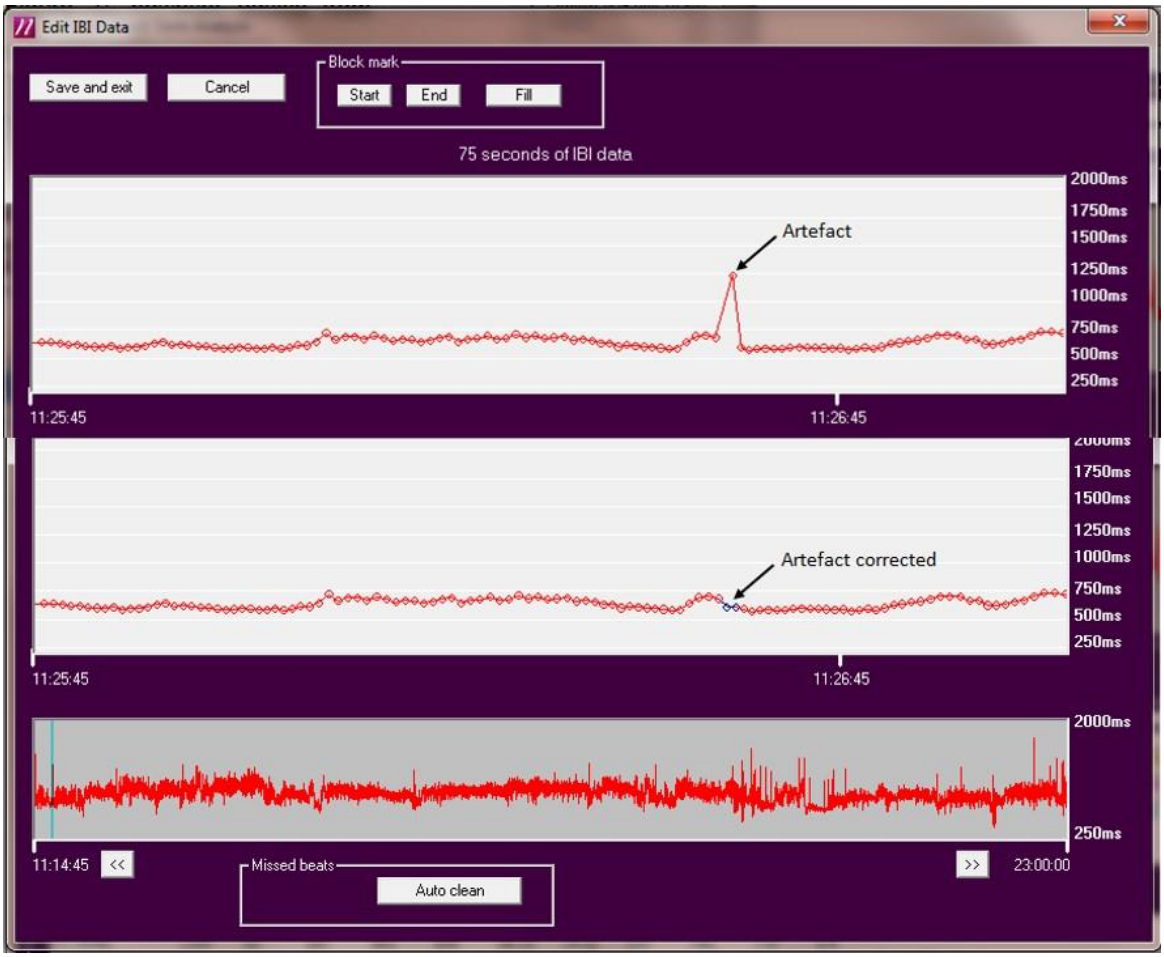
After fitting the Actiheart device, a short signal test, involving a 5 minute walk with the participant, was performed to confirm that the level of R wave signal being picked up by the device was adequate to avoid artefacts due to either high noise level or a low R wave signal. The test involves making a short recording and analysing the signal using a built-in utility called Signal Test which provides a Pass/Fail indication of whether the signal is acceptable. During the signal test, once the subject has worn the Actiheart for the required length of time, data was downloaded to the laptop and analysed for the quality of the signal. Any areas of the recording that failed the test were displayed in red on the scan. If a recording has failed overall, it usually means that either the noise level was too high or the R wave was too low. Once an acceptable signal was obtained, a short-term recording[2] was programmed and the monitors would be worn for 24 h. During the 24 h recording period, the ECG signal is electronically amplified with the resulting ECG signal sampled at 125 Hz. QRS detection is difficult, not only because of the physiological variability of the QRS complexes, but also because of the various possible types of noise in the ECG signal, including muscle noise and artefacts due to electrode motion. To detect the slope of the R wave in order to locate the QRS complex, a real time QRS detection algorithm is used (190). The Actiheart firmware then applies a detection threshold whose sensitivity is dependent on the amount of physical movement detected by the Actiheart. After completing the 24 h recording, data processing of the 24 h recordings was carried out to remove 'noisy' data and artefacts and it was performed in three stages. Firstly, the data was downloaded to the Actiheart software (version 4.0.91, CamNtech Ltd, Cambridge, UK) to isolate the RR recordings and manually remove any missed beats. Once edited, the time-stamped raw IBI data was exported to Kubios HRV analysis software (Biosignal Analysis and Medical Imaging Group, Department of Physics, University of Kuopio, Finland) to apply further artefact corrections. Finally, after all amendments to the data have been made, the HRV data was compiled in a Microsoft Excel spreadsheet.

[2] "Short term recording" mode allows recording of up to 440,000 heartbeats. A "Long term recording" can last between 11 and 21 days depending on set-up.

Data collection from the Actiheart device was fixed at 15 s epochs, but for post hoc analysis of time domain and frequency domain indices of HRV, 5 min epochs were selected to obtain parameters that reflect the circadian and longer term fluctuations. At the end of each epoch the RR interval durations for the last 16 IBIs are analysed to get a representative value and remove some of the main artefacts. The 16 IBI values are averaged and any RR intervals greater than +/- 25% of this average are removed. Finally, the remaining RR intervals are averaged and converted to beats per minute.

The HRV parameters more sensitive to artefacts are the frequency domain measures and RMSSD (74). The artefacts can be caused by a lack of signal detection due to a loss of contact between the electrode and skin and it will show up as a missed beat (large spike upwards) on the tachogram. Although some of the artefacts are removed automatically by the Actiheart software, this software has a limited scope for artefact correction, which leaves most artefacts to be manually corrected using the Actiheart edit IBI data function. Missed beats where the apex of the spike was ≥ baseline + 500 ms were manually corrected as illustrated in **Figure 9**.

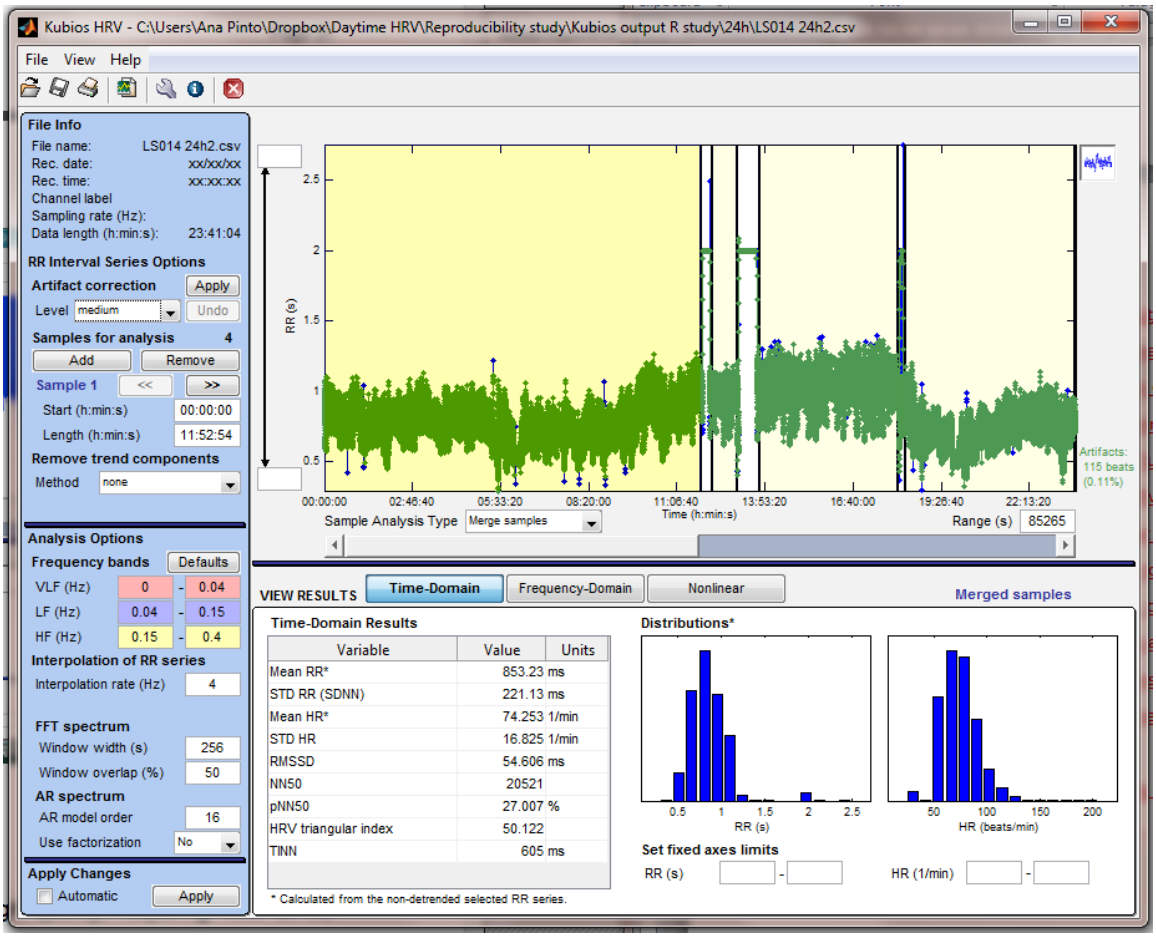
Figure 9 – Missed beats manual correction by double clicking on the artefact.



After manually removing the artefacts with the Actiheart software and saving the corrections, edited time-stamped raw IBI data was imported to Kubios software which has an artefact correction function with different levels of correction that range between very low to very strong. Because the artefact correction uses interpolation to yield the missing or corrupted data, distortion of the results might occur at high correction levels. For this reason, a low correction was chosen to be applied to all data files.

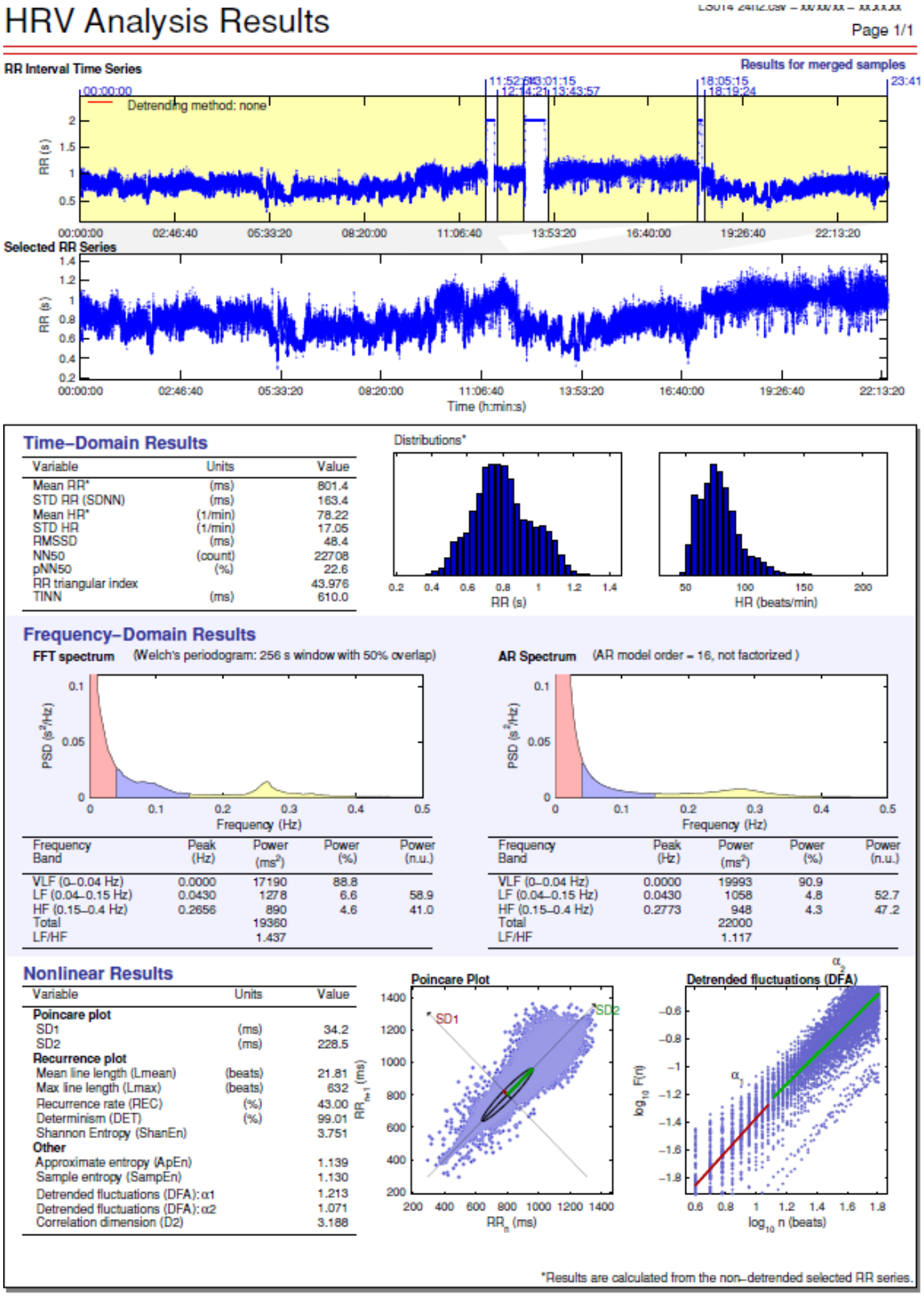
When files contained sections of poor quality data (identified with the Actiheart software), removal of these sections was performed in Kubios and data was merged to give the final figures (**Figure 10**).

Figure 10 – Removal of poor quality data using Kubios software. Figure also shows a medium correction selected.



After applying all corrections, the file was saved in a pdf and text form that allowed the transcription of the values of the HRV parameters of interest into an Excel spreadsheet for further analysis (**Figure 11**).

Figure 11 – Kubios output file showing the time domain, frequency domain (FFT spectrum only) and nonlinear (Poincaré plot only) HRV parameters results.

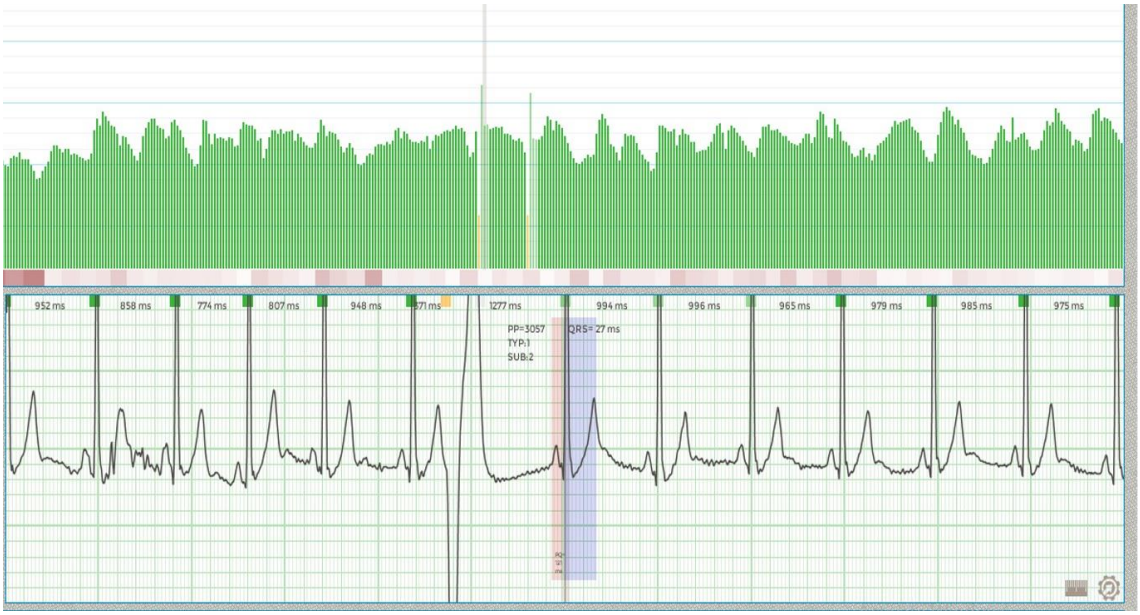


2.1.4. Recording of the IBI and HRV analysis using the eMotion Faros device

Cardiscope™ analytics (HASIBA Medical GmbH) is the software application used to program the eMotion Faros monitor and analyse the ECG data in order to obtain HR/IBI and HRV measurements. There are two recording modes, an online mode with real-time transmission operated by Bluetooth, and a datalogger mode to record ECG data offline. The online mode was used to visually examine the ECG recording in real-time to confirm that the monitor was picking up a good signal. Once a good signal was confirmed the measurements taken during the Met-IER study visits (resting and mental stressor tests) were performed in this mode. The offline mode was used to record 24 h ambulatory data. The ECG sampling rate was fixed at 250 Hz for both modes. The Cardiscope software automatically removed artefacts on a beat-to-beat basis, as well as any ventricular or ectopic beats, from the analysis. Each tachogram - depicting consecutive distances between R waves of the QRS complexes - alongside the ECG recording would be visually inspected (**Figure 12**) to ensure that no extra processing was needed. In the rare event of having to further process the ECG data this was done manually. **Figure 13** illustrates the detection of an ectopic beat and automatic removal from the analysis by the software.

Figure 12 – Visual inspection of tachogram (top) with ECG recording. Each green bar represents one IBI, measured on the ECG recording between green squares.

Figure 13 – Detection of the ectopic beat in the ECG recording (orange) by the software and automatic removal from the analysis (shaded colour in the tachogram).



Once data was inspected, episodes were added to the tachogram in order to assign the following activities/periods: resting, mental stressor test, sleep-time, day-time and 24 h. HRV parameters were then calculated for each episode and data was exported directly to an Excel file.

2.1.5. Actiheart vs. eMotion Faros

The Actiheart monitor was very well tolerated by the participants, and most of them would even forget they were wearing it, so participant burden was close to null. The Actiheart device is waterproof allowing participants to shower, although under water the noise level is so high that data quality becomes poor and need to be removed from the analysis. One of the main concerns with the Actiheart recordings relates to the HRV data analysis which includes a substantial amount of manual data editing and processing that is needed to provide the final results. This may potentially introduce observer error and bias. Three types of ECG pads were tested in the reproducibility study and the ones that lasted at least 24h without falling off and had a better signal were the 4841P (Ambu®). Nevertheless, it was still difficult to obtain a good signal/noise ratio on a minority of participants, and these tended to be those with the lowest % body fat or BMI. Some loss-of-signal problems arose during sleep-time. It was concluded that better ECG pads could be helpful for minimising the amount of further manual data processing

so new ECG pads were tested with SP-50 (50 mm round, Pulse Medical) providing a better signal and lasting longer on the skin. For this reason the vegan, renal and Met-IER study used the Pulse Medical SP-50 ECG pads. Although the manual processing and editing of HRV analysis might introduce some human error, the same person analysed all the data reducing inter-individual error. Also, random recordings were selected to be analysed separately by Dr. Wendy Hall and the results were similar, showing a good degree of consistency between individuals.

Actiheart measures HRV through the IBI, collecting only the time between QRS complexes and discarding the ECG recording, whereas the eMotion Faros measures HRV through ECG recordings, which is more accurate in terms of artefact correction because it is possible to see every QRS complex of the recording. Also, the Actiheart software is more likely to introduce errors as there is no ECG raw data that could be inspected to check for ectopic or ventricular beats. Also, when excluding noisy or poor quality data in the Kubios software, this is done by removing periods of data, as opposed to the Cardioscope software where data is excluded on a beat-to-beat basis, allowing more precise and accurate data processing. In addition, the recording quality assessed by the percentage of good/valid data in each recording was superior using the eMotion Faros monitor compared to the Actiheart.

2.2. Blood pressure

2.2.1. Seated blood pressure

Seated blood pressure was measured three times with an A&D Medical UA-767Plus (San Jose, USA) upper arm automated blood pressure monitor with an appropriate cuff size in accordance with guidelines from the BHS. While taking the blood pressure measurements the participant was sitting with the legs uncrossed and feet flat on the floor, calm and instructed not to talk. The arm used to take the measurement was relaxed and supported by the table and the cuff was wrapped around the upper arm allowing space for a finger between the participant's arm and the cuff. Three readings were taken, the first one was discarded and the other two were averaged.

2.2.2. Ambulatory blood pressure

Ambulatory blood pressure (ABP) was measured using an upper arm A&D Medical TM-2430 ABP monitor with an appropriate cuff size fitted to the non-dominant arm. When asked to wear the ABP monitor for 24 h, the subjects were shown how to adjust the cuff in case of movement and the monitor was programmed to automatically inflate the cuff and take readings every 30 minutes during the day (7am to 10pm) and hourly at night (10pm to 7am). The sleep time was adjusted for each participant according to the reported sleep hours in the activity diary. Once the monitors were returned, data was downloaded and any misreading or physiological anomalies were discarded in order to obtain the mean 24 h, day/awake time and sleep-time SBP and DBP data.

2.3. Erythrocyte membrane and plasma fatty acid analysis

Plasma fatty acid composition was determined based on the Lepage and Roy method (191), substituting toluene for benzene and using pentadecanoic acid as an internal standard. Briefly, a toluene/methanol solution was prepared with a ratio of 1:4 respectively by volume. The solution was then acidified by the addition of acetyl chloride (10% of initial vol) to generate the methylating reagent. A 100 µl aliquot of plasma was dispensed into a glass tube. This was followed by the addition of 2.2 ml of the methylating agent. Following incubation of the mixture at 60 ºC for 2 h to effect the formation of phosphoglycerides to fatty acid methyl esters (FAME), the mixture was neutralised with 5 ml of 6% w/v aqueous sodium carbonate. The resulting mixture was then subjected to centrifugation to effect phase separation. Finally, the FAME containing supernatant was aspirated off and transferred to a vial for gas chromatography (GC) analysis.

Erythrocyte membrane phospholipid fatty acid composition was determined as previously described (192). A 2 ml aliquot was withdrawn from the glass tube and transferred to a glass vial for evaporation to dryness using a compressor for 1 h at 65 ºC. The resulting dried residue was reconstituted by adding 200 µl of hexane, followed by the addition of 100 µl of sodium methoxide in methanol solution (with calcium chloride as a drying agent). The resulting mixture

was incubated for 2 h to allow the transesterification of the FAME. Following the incubation, the supernatant was collected and transferred to insert vials to perform further analysis by GC.

The FAME mixtures obtained from plasma and erythrocyte membranes were injected into a gas chromatograph (Agilent Technologies 6890) using H2 as a carrier gas flow set at 1 ml/min equiped with a flame ionization detector set at 250 ºC. The column used for the analyses was a 25 m lenght x 0.22 mm internal diameter x 0.25 µm film thickness BPX70 capillary column (SGE, Melbourne, VIC, Australia).

For the plasma FAME mixture, the injection was performed in split mode (50:1) and for the erythrocyte membrane FAME mixture the injection was performed in splitless mode, both with the inlet maintained at 240 ºC. Temperature programming was employed to optimise the separation. For the plasma FAME mixture, this was achieved by holding the initial column temperature at 160 ºC for 4 minutes (80 ºC for 2 minutes for the erythrocyte FAME mixture), followed by ramping at 10 ºC/min (15 ºC/min for the erythrocyte FAME mixture) to 200 ºC and holding at this temperature for 10 minutes to ensure complete elution. Fatty acids were identified by matching their retention times with those present in the reference standards run under identical conditions. Proportions of fatty acids as weight % were quantified by expressing the areas under the chromatographic peaks as a % of the total integrated area.

2.4. Anthropometry

2.4.1. Height

Height was measured using a wall mounted stadiometer and recorded to the nearest 1 mm while participants were barefoot, including removal of socks, and standing upright with feet together. Heels, buttock and shoulders were against the measuring rod and arms were hanging freely with palms facing the thigh. The measurement was taken after the participant inhaled deeply while looking straight ahead.

2.4.2. Weight and percentage of body fat

Weight and percentage of body fat were measured using a TANITA BC-418 segmental body composition analyser and recorded to the nearest 0.1 kg and 0.1%, respectively. Participant were barefoot, wearing light clothing (one layer of clothes) and were asked to empty their pockets and remove any accessories such as watches and rings.

2.4.3. Waist and hip circumference

A non-stretch measuring tape was used to measure waist and hip circumference to the nearest 0.1 cm while the participant was standing up right and breathing normally. Weight was evenly balanced on both feet, which were set apart equivalent to the shoulder width. The waist measurement was taken at the midway point between the iliac crest and the lowest rib with the participant's waist uncovered or with very light clothing. For the Met-IER study, the waist circumference was measured around the umbilicus due to the overweight/obese and multi-ethnicity nature of the population (193) and the fact that waist circumference thresholds for detecting high-risk of cardiometabolic disease were shown to be more sensitive at the level of the umbilicus (194). The hip circumference was taken at the widest point over the buttocks and below the iliac crest.

Chapter 3 Methods development

This chapter consists of the work done in order to optimise and develop the HRV recording and analysis methods as well as provide further guidance for the primary PhD studies. Two studies are presented: the reproducibility study and the MARINA study. Results and considerations obtained from these studies used for planning the primary PhD studies are also presented.

3.1 Reproducibility study

3.1.1. Introduction

Measuring HRV in free-living conditions is more challenging as opposed to measuring it under controlled conditions. As the free-living state will be of great importance in the doctoral studies presented in this thesis, it is important to explore the reproducibility of ambulatory HRV measurements and optimise the method used for collection and analysis before finalising the study design of the primary research studies. The free-living carries various challenges as it is not controlling for numerous factors that could potentially impact HRV such as physical activity levels. In a large prospective study in older adults, physical activity was independently associated with 24 h SDNN (195). In addition, a meta-analysis of intervention studies showed that aerobic physical activity significantly increased resting IBI and HF power in adults (136). For this reason, the effect of physical activity levels on HRV in the free-living will also be explored in this study.

3.1.2. Study design and aims

This was an observational cross-sectional study conducted at King's College London (KCL) between January and May 2013 that aimed at investigating the reproducibility of the Actiheart monitor to assess HRV in free-living conditions among healthy subjects, by exploring the intra- and inter-variability of the different HRV parameters. A secondary aim included testing different types of pads to choose the best one for duration and signal/noise ratio. These included Ambu® 4841P short term ECG monitoring electrodes, Bio Prothec Inc. TELECTRODE T815 and Unilect^{TM} 6110M hypoallergenic electrodes. Ethical approval was

obtained from KCL research ethic committee (BDM/12/13-37) and written informed consent was given by participants.

3.1.3. Methods

Twenty-four healthy men and women aged over 18 years volunteered to take part in this study. Those with a medical history of CVD (including atrial fibrillation), diabetes, cancer (excluding basal cell carcinoma), renal, liver, inflammatory bowel disease, history of substance abuse or alcoholism, pregnancy, and BMI > 35Kg/m2 were excluded. The volunteers were mostly PhD students and some research staff. Participants wore the Actiheart monitor for 3 to 4 days (24 hours each). In the first study visit, standard measurements were made including height, weight, waist circumference, body composition by bioelectrical impedance to estimate % body fat, and seated blood pressure according to the guidelines of the British hypertension society (BHS). Volunteers were then fitted the Actiheart device in the morning period and were asked to wear it for 24 h. A diary was provided during the recording periods to keep a record of all the daily activities (activities/exercise, meals or naps). After 24 h of each visit participants were asked to attend the Metabolic Research Unit (MRU) for a 5-10 min visit to remove and return the device. Volunteers were asked to refrain from eating, drinking (except water) and vigorous exercise for at least 2 hours before they arrive at the research facility. HRV, HR/IBI and accelerometry data were analysed for the full length of recording time (minimum of 18 h, up to 24 h). HRV outcomes included time and frequency-domain parameters.

3.1.4. Results

Figure 14 shows the number of participants completing HRV measurements ranging from 1 to 4 days of measurements. Participants excluded from the analysis were due to not having a satisfactory 24 h recording, which includes at least 18 h of good quality data. Participants completing 4 days with good recordings were used to assess inter-individual variability and the full sample with at least one good recording was used to assess intra-individual variability (n=22).

Figure 14 - Participants distribution according to the number of recordings completed and number of good recordings.

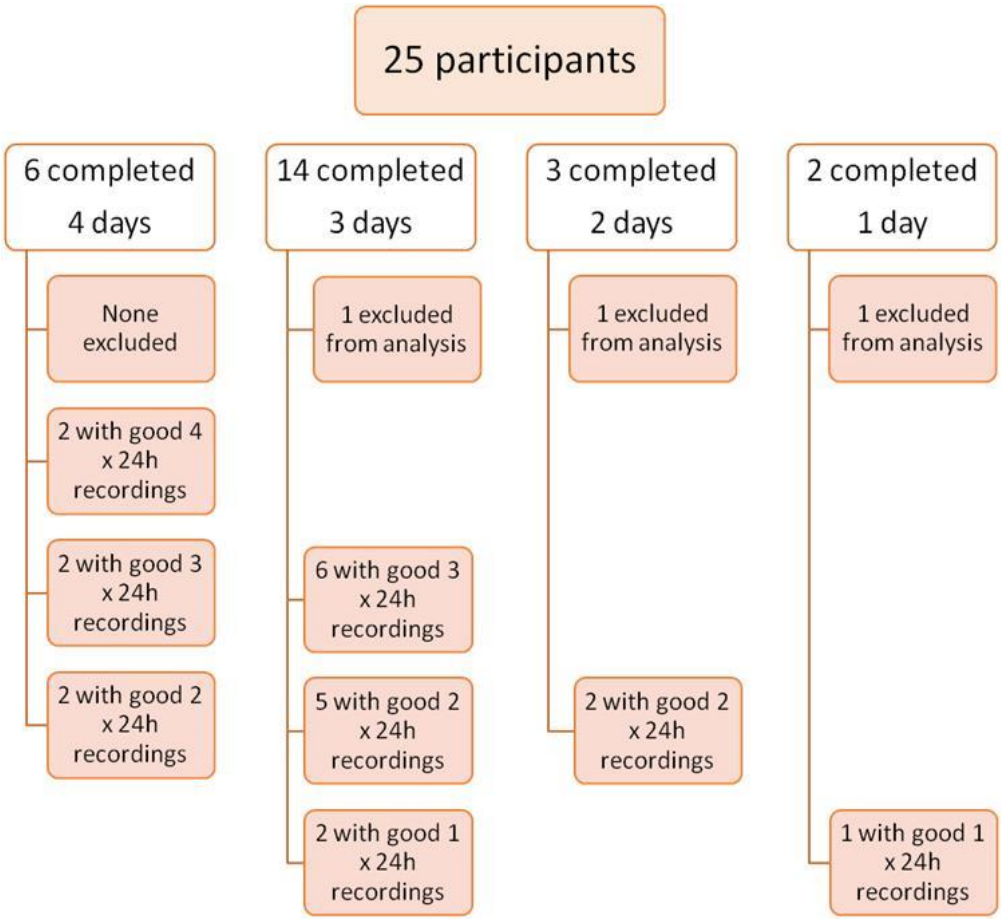


Table 5 shows the characteristics of the 22 subjects that were included in the analysis. There were 5 males and 17 females with a mean age of 23.8 ± 2.3 and 27.1 ± 5.3 years, respectively. Participants presented a normal BMI (indicative of healthy weight: 18.5-24.9 kg/m^2 (196)), % body fat, resting systolic blood pressure (SBP), diastolic blood pressure (DBP), and HR.

Table 5 - Subjects' characteristics who presented one to four good 24h recordings (n=22).

	Male (n=5)	**Female (n=17)**
Age (years)	23.8 ± 2.3	27.1 ± 5.3
BMI (kg/m^2)	22.0 ± 3.2	21.0 ± 3.0
% body fat	10.8 ± 5.7	24.3 ± 5.6
Resting SBP (mmHg)	118 ± 8	99 ± 13
Resting DBP (mmHg)	76 ± 7	71 ± 10
Heart Rate (bpm)	71 ± 15	73 ± 10

Results are expressed as mean ± SD. BMI, body mass index; bpm, beats per minute DBP, diastolic blood pressure; SBP, systolic blood pressure.

3.1.4.1. Intra-individual variability

To assess the impact of physical activity variability on HRV in the free living, an example of a participant that has different activity levels in three different days was presented in comparison to a participant that kept activity levels similar throughout 3 days. **Table 6** shows the CVs of the different HRV parameters from two different participants with different physical activity variability on three different days. Participant 1 presents high physical activity variability and hence **Table 6** displays 3 days of different activity levels (low, medium and high). Participant 2 presents low physical activity variability and hence **Table 6** displays 3 days of similar activity levels. Different levels of physical activity had a considerable influence on the variability of the frequency domain parameters (LF, HF, LF : HF ratio and VLF) as shown by the higher CVs and the triangular index, as well as doubling the variance of the IBI and HR.

Table 6 – Impact of physical activity levels in 24 h free-living HRV monitoring.

HRV parameters	**Activity level**			
Participant 1 High physical activity variability	Low	Medium	High	CV (%)
Activity (cpm)	155	326	523	55.0
HR (bpm)	79	81	83	2.3
IBI (ms)	782	779	751	2.3
SDNN (ms)	133	145.2	148	5.8
SDANN(ms)	109	123.3	118	6.3
pNN50 (%)	26.5	25.4	28.5	5.8
RMSSD (ms)	53.9	50.9	60.9	9.3
Ti	26.5	31.4	45.0	27.9
LF (ms^2)	1608	1633	2189	18.2
HF (ms^2)	1211	1112	1750	25.3
LF:HF ratio	1.33	1.47	1.25	8.2
VLF (ms^2)	8530	10479	13190	21.8
SD1	38.1	36.0	43.1	9.3
SD2	184	202	205	5.8
SD1:SD2 (Poincaré point)	0.21	0.18	0.21	9.0
Participant 2 Low physical activity variability	Low	Low	Low	CV (%)
Activity (cpm)	242	206	209	9.2
HR (bpm)	70	64	69	4.8
IBI (ms)	946	1015	936	4.5
SDNN (ms)	293	278	263	5.3
SDANN(ms)	268	248	237	6.2
pNN50 (%)	37.3	40.5	34.8	7.6
RMSSD (ms)	88.7	100.7	88.9	7.4
Ti	57.7	58.7	46.8	12.1
LF (ms^2)	2915	3542	2937	11.4
HF (ms^2)	2816	3364	2826	10.4
LF:HF ratio	1.04	1.05	1.04	0.9
VLF (ms^2)	59493	49999	49172	10.8
SD1	62.7	71.2	62.9	7.4
SD2	409	387	367	5.5
SD1:SD2 (Poincaré point)	0.15	0.18	0.17	9.2

CV, coefficient of variation; HF, high frequency; IBI, interbeat interval; LF, low frequency; pNN50, percentage of adjacent normal-to-normal intervals that differed by >50ms; RMSSD, root mean square of successive differences of normal-to-normal intervals; SD1, standard deviation of the Poincaré plot perpendicular to the line of indentity; SD2, standard deviation of the Poincaré plot along the line of indentity; SDANN, standard deviations of the average NN intervals; SDNN, mean of the standard deviations of the normal-to-normal intervals; Ti, triangular index; VLF, very low frequency.

3.1.4.2. Inter-individual variability

Table 7 shows the results from the inter-individual variability of the different HRV parameters according to sex, and in the whole study sample. The most robust measurements - the ones with least variability - were HR and IBI. However, the HRV parameters presented a high inter-individual variability which means that these parameters might be correlated with other factors, such as dietary factors or biomarkers, that haven't been measured in this reproducibility study but will be measured in the main studies of this doctoral research.

Table 7 – HRV parameters of the whole study sample – inter-individual variability.

HRV parameters	Male (N=5)	Female (N=17)	Total (N=22)	CV (%)
Activity (cpm)	405 ± 191	214 ± 71	249 ± 122	49
HR (bpm)	81.6 ± 7.0	81.1 ± 5.9	81.2 ± 5.9	7.3
IBI (ms)	798 ± 71	779 ± 69	783 ± 68	8.7
SDNN (ms)	213 ± 33	160 ± 39	170 ± 43	25
SDANN (ms)	192 ± 33	141 ± 36	150 ± 40	27
pNN50 (%)	22.0 ± 5.6	21.0 ± 9.7	21.2 ± 9.0	43
RMSSD (ms)	50.7 ± 8.9	57.9 ± 26.5	56.6 ± 24.3	43
Ti	55.6 ± 14.8	41.7 ± 8.9	44.2 ± 11.2	25
LF (ms^2)	2033 ± 882	1370 ± 734	1491 ± 827	55
HF (ms^2)	930 ± 313	1072 ± 734	1055 ± 673	64
LF:HF ratio	2.28 ± 0.82	1.53 ± 0.63	1.63 ± 0.69	43
VLF (ms^2)	30699 ± 9259	17177 ± 10222	19636 ± 11195	57
SD1	35.8 ± 6.3	40.9 ± 18.8	21.2 ± 9.0	43
SD2	299 ± 47	222 ± 54	236 ± 60	26
SD1:SD2 (Poincaré point)	0.12 ± 0.01	0.18 ± 0.07	0.17 ± 0.07	38

Results are expressed as mean ± s.d.
CV, coefficient of variation; HF, high frequency; IBI, interbeat interval; LF, low frequency; pNN50, percentage of adjacent normal-to-normal intervals that differed by >50ms; RMSSD, root mean square of successive differences of normal-to-normal intervals; SD1, standard deviation of the Poincaré plot perpendicular to the line of indentity; SD2, Standard deviation of the Poincaré plot along the line of indentity; SDANN, standard deviations of the average NN intervals; SDNN, mean of the standard deviations of the normal-to-normal intervals; Ti, triangular index; VLF, very low frequency.

3.1.5. Discussion

The results of this study were important to help define the design of subsequent studies since it provided an insight of the difficulties associated with measuring HRV in free living conditions, namely the high inter- and intra-individual variability presented. This study helped define some of the variables that influence HRV readings including sex and physical activity. A recent meta-analysis on sex differences in HRV (197) showed that time-domain parameters were significantly lower in female subjects compared to male subjects, but females presented a

lower LF : HF ratio, which agree with this reproducibility study results. The marked variability observed may not always reflect sympathetic and parasympathetic activity during day-to-day activities if not controlled for potential confounders. For the studies measuring HRV in free living conditions described in this thesis, special care was taken such as asking participants to avoid any strenuous physical activity as well as including sex as a fixed factor and physical activity as a covariate in the statistical analysis. Other factors that influence HRV have been reported in studies, including BMI (126), age (198,199), smoking (200,201), acute alcohol intake (202). For this reason, the design of the next studies excluded smokers and matched participants according to age and BMI, as well as asking participants to refrain from alcohol the day before HRV measurements took place.

3.2 MARINA Study

The MARINA (Modulation of Atherosclerosis Risk by Increasing dose of N-3 fatty Acids) study was a single-centre RCT to investigate the dose-response effect of fish oil supplementation (provided as purified triacylglycerol with an EPA:DHA ratio of 1.5:1 and supplied by Croda Chemicals Europe Ltd, Hull, Yorkshire, UK) at doses relevant to dietary intakes on cardiovascular risk factors, conducted at KCL between April 2008 and October 2010. The MARINA study collected Actiheart data on HRV as a secondary outcome. Detailed methods and primary outcomes have been reported previously (203). Sleep-time HRV data were previously analysed and results were published by Dr. Hall and colleagues (186), showing significant increases in SDANN, Ti and VLF power with LC *n*-3 PUFA supplementation, an effect which was not related to dose. However, the day-time data had not yet been analysed due to the time-consuming nature of data cleaning/processing raw data from a large trial. HRV analysis of the MARINA day-time IBI data therefore formed part of the HRV analysis training for the PhD candidate and is presented in the methods chapter as it contributed to further development of the methods used for Acitheart HRV analysis and the information gathered was used in the planning of the subsequent study (OM3GA Study - Chapter 4).

3.2.1 Methods

The MARINA trial was a parallel design, double-blinded, RCT that compared the intake of 0.45, 0.9 and 1.8 g of EPA + DHA-rich triacylglycerols provided in 3 soft gel capsules (Croda Chemicals Europe Ltd, Hull, Yorkshire, UK) per day with matched placebo capsules containing an oleic acid-rich triacylglycerol consumed for 12 months. The EPA : DHA ratio was 1.5:1 and doses were chosen in order to be equivalent to eating 0 (placebo), 1, 2 or 4 portions of oily fish a week. Ethical approval was obtained by the St Thomas' Hospital NHS Research Ethics Committee (ref: 08/H0802/3) and was registered at www.controlled-trials.com (ref: ISRCTN66664610). Men and women, non-smokers, aged 45 to 70 years attended a screening visit and those with a medical history of CVD (including atrial fibrillation), type 1 diabetes mellitus, uncontrolled type 2 diabetes (fasting plasma glucose > 7 mmol/L), cancer (excluding basal cell carcinoma) in the past 5 years, chronic renal, liver or inflammatory bowel disease, history of substance abuse or alcoholism, pregnancy, weight change > 3 kg in the preceding 2 months and BMI < 20 and > 35 kg/m^2 were excluded. Potential eligible participants were asked to complete a 4 week run-in period taking placebo capsules and restricting oily fish intake. Once the run-in period was completed, participants were randomised to treatment by minimisation to balance age, gender and ethnicity. The day before each study visit, participants were instructed to follow standardised dietary advice, as well as to fast overnight for 12h and to refrain from any strenuous physical activity. Outcome measurements, including 24 h ambulatory HRV (using the Actiheart monitor), were taken at baseline (after completing the run-in period), after 6 and 12 months of the dietary intervention. Day-time data was analysed on an intention to treat basis based on the average values on treatment (mean of 6 and 12 months) using analysis of covariance (ANCOVA) adjusted for baseline values as well as for physical activity, age, gender, ethnicity and BMI. Recordings that presented < 6 h of good quality data were excluded. Data normality was determined using Shapiro-Wilk test and by visual inspection of the data distribution on the histogram and Q-Q plots. Non-normally distributed data were normalised by natural logarithm (LN) before analysis. This was a time-consuming analysis as there were 305 participants with 3 time points, which translates into 915 recordings to process. Depending on the quality of data, i. e. the amount of data needing manual editing and noisy data

that needed to be cut from the tachograms, the analysis of each recording took between 15 mins to over an hour to process, with an average of 40 mins per recording. The data processing took place between December 2012 and March 2014.

3.2.2 Results

Of 367 participants randomised to a treatment group, 305 participants completed the ambulatory 24 h HRV recordings and after processing the data, 257 participants presented usable day-time data for all three visits (mean 11:53 h; range 8:04, 15:11). **Table 8** presents the subjects' characteristics of the participants included in the HRV analysis according to the allocated treatment group.

Table 8 - Subjects' characteristics at baseline according to treatment group (n=257).

	Placebo (n=57)	**0.45 g/d (n=71)**	**0.9 g/d (n=64)**	**1.8 g/d (n=65)**	***p* value** [†]
Age (years)	55. 6 ± 7.1	55.0 ± 7.0	55.5 ± 6.4	55.0 ± 6.7	0.921
Sex					
Male	23 (40)	29 (41)	22 (34)	26 (40)	0.862 [§]
Female	34 (60)	42 (59)	42 (66)	39 (60)	
Ethnicity					
White	50 (87.7)	56 (78.9)	53 (82.8)	57 (87.7)	0.244 [§]
Black	4 (7.0)	4 (5.6)	4 (6.3)	1 (1.5)	
Asian	1 (1.8)	6 (8.5)	6 (9.4)	1 (1.5)	
Far Eastern	0 (0)	2 (2.8)	0 (0)	3 (4.6)	
Other	2 (3.5)	3 (4.2)	1 (1.6)	3 (4.6)	
BMI (kg/m^2)	26.0 ± 3.6	25.4 ± 3.8	26.1 ± 4.0	25.2 ± 3.4	0.470
Resting SBP (mmHg)	122 ± 17	120 ± 14	123 ± 14	120 ± 14	0.432
Resting DBP (mmHg)	77 ±10	77 ± 10	78 ± 8	76 ± 9	0.480
Erythrocyte EPA (%)[a]	1.26 (1.16, 1.37)	1.19 (1.10, 1.29)	1.29 (1.18, 1.42)	1.22 (1.12, 1.33)	0.542
Erythrocyte DHA (%)	6.58 (6.24, 6.91)	6.47 (6.17, 6.76)	6.30 (5.89, 6.72)	6.55 (6.17, 6.93)	0.712
Omega-3 index (%)	7.91 (7.48, 8.33)	7.73 (7.37, 8.09)	7.69 (7.19, 8.19)	7.85 (7.38, 8.31)	0.895

BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Results are expressed as n (%) or mean ± SD or mean (95% CI), except [a] Geometric mean (95% CI). [†] comparison between treatment groups by one-way ANOVA, except [§] χ^2 test.

Table 9 shows the HR, IBI and HRV parameters at baseline and the effect of following a dietary intervention with increasing doses of LC *n*-3 PUFA versus placebo. There was a significant treatment effect in SDANN and VLF after supplementation with LC *n*-3 PUFA. These results were not dose-dependent as the strongest effects were seen after supplementation with 0.9 g per day but not 0.45 g and 1.8 g of EPA + DHA per day.

Table 9 - HR, IBI and HRV parameters at baseline and following supplementation with increasing doses of LC n-3 PUFA.

HRV parameters		Placebo (n=57)	0.45 g/d (n=71)	0.9 g/d (n=64)	1.8 g/d (n=65)	*p* value
HR (bpm)	Baseline	86.1 (81.0, 91.2)	83.7 (79.8, 87.6)	81.8 (77.3, 86.3)	87.1 (82.1, 92.1)	
	Average on treatment[a]	83.8 (80.8, 86.8)	81.2 (78.9, 83.6)	82.5 (79.9, 85.1)	81.9 (79.0, 84.8)	
	Treatment effect*	1.000 [reference]	0.970 (0.942, 0.997)	0.984 (0.953, 1.015)	0.977 (0.942, 1.012)	0.597
IBI (ms)	Baseline	719 (675, 765)	738 (703, 775)	759 (718, 802)	706 (664, 751)	
	Average on treatment[a]	741 (714, 769)	762 (741, 786)	745 (721, 770)	758 (731, 787)	
	Treatment effect*	1.000 [reference]	1.004 (1.000, 1.009)	1.001 (0.996, 1.006)	1.003 (0.998, 1.009)	0.550
SDNN (ms)	Baseline[a]	98.0 (85.1, 113.0)	98.1 (88.0, 109.4)	118.6 (104.6, 134.4)	100.6 (87.5, 115.6)	
	Average on treatment[a]	110.1 (100.4, 120.6)	114.9 (106.9, 123.4)	100.1 (92.3, 108.5)	114.3 (104.5, 125.1)	
	Treatment effect*	1.000 [reference]	1.009 (0.994, 1.024)	0.980 (0.963, 0.997)	1.008 (0.989, 1.027)	0.064
SDANN (ms)	Baseline[a]	82.0 (70.0, 96.2)	81.8 (72.3, 92.4)	105.3 (91.5, 121.2)	84.3 (72.0, 98.5)	
	Average on treatment[a]	94.4 (84.6, 105.3)	97.8 (89.8, 106.7)	83.6 (75.9, 92.2)	100.9 (90.6, 112.3)	
	Treatment effect*	1.000 [reference]	1.008 (0.989, 1.027)	0.973 (0.952, 0.995)	1.015 (0.991, 1.038)	**0.048**
RMSSD (ms)	Baseline	14.6 (11.1, 19.3)	18.7 (15.1, 23.0)	21.4 (16.8, 27.3)	16.0 (12.2, 20.9)	
	Average on treatment[a]	18.5 (15.6, 22.1)	18.5 (16.1, 21.2)	15.2 (13.0, 17.7)	18.2 (15.3, 21.7)	
	Treatment effect*	1.000 [reference]	0.999 (0.951, 1.046)	0.932 (0.878, 0.985)	0.994 (0.935, 1.053)	0.215
pNN50 (%)	Baseline	2.1 (1.1, 4.2)	3.2 (1.9, 5.5)	3.6 (2.0, 6.4)	2.8 (1.4, 5.4)	
	Average on treatment[a]	3.8 (2.5, 5.9)	4.2 (3.0, 5.9)	2.4 (1.6, 3.6)	3.7 (2.4, 5.7)	
	Treatment effect*	1.000 [reference]	1.067 (0.810, 1.325)	0.658 (0.369, 0.948)	0.982 (0.660, 1.304)	0.185
Ti	Baseline	24.4 (20.9, 28.3)	25.2 (22.4, 28.3)	32.6 (28.6, 37.3)	27.1 (23.3, 31.4)	
	Average on treatment[a]	30.1 (27.0, 33.6)	31.2 (28.7, 34.0)	26.4 (24.0, 29.1)	29.7 (26.7, 33.0)	
	Treatment effect*	1.000 [reference]	1.010 (0.985, 1.035)	0.962 (0.934, 0.990)	0.996 (0.965, 1.027)	0.080
HF (ms^2)	Baseline[a]	125 (78, 201)	142 (98, 204)	180 (117, 272)	169 (106, 270)	
	Average on treatment[a]	167 (118, 234)	198 (152, 259)	136 (100, 183)	176 (126, 246)	
	Treatment effect*	1.000 [reference]	10.34 (0.982, 1.086)	0.960 (0.901, 1.019)	1.011 (0.945, 1.076)	0.317

LF (ms^2)	Baseline[a]	396 (286, 549)	412 (320, 529)	570 (427, 761)	521 (378, 718)	
	Average on treatment[a]	489 (403, 594)	536 (460, 624)	437 (369, 519)	501 (414, 606)	
	Treatment effect*	1.000 [reference]	10.15 (0.990, 1.039)	0.982 (0.954, 1.010)	1.004 (0.973, 1.034)	0.377
LF:HF ratio	Baseline[a]	3.17 (2.19, 4.58)	2.90 (2.19, 3.85)	3.19 (2.31, 4.42)	3.08 (2.14, 4.42)	
	Average on treatment[a]	3.06 (1.48, 3.77)	2.78 (2.35, 3.27)	3.41 (2.83, 4.10)	3.25 (2.65, 4.00)	
	Treatment effect*	1.000 [reference]	0.914 (0.767, 1.062)	1.098 (0.932, 1.263)	1.056 (0.872, 1.241)	0.395
VLF (ms^2)	Baseline[a]	5973 (4476, 7972)	6229 (4990, 7776)	8911 (6902, 11492)	6542 (4926, 8690)	
	Average on treatment[a]	8973 (7448, 10804)	8553 (7391, 9896)	6549 (5558, 7715)	8392 (6995, 10075)	
	Treatment effect*	1.000 [reference]	0.995 (0.979, 1.011)	0.965 (0.947, 0.983)	0.993 (0.973, 1.013)	**0.042**
SD1:SD2	Baseline[a]	0.12 (0.09, 0.15)	0.13 (0.11, 0.15)	0.11 (0.09, 0.13)	0.12 (0.10, 0.15)	
(Poincaré ratio)	Average on treatment[a]	0.12 (0.10, 0.14)	0.12 (0.11, 0.14)	0.11 (0.10, 0.13)	0.11 (0.10, 0.13)	
	Treatment effect*	1.000 [reference]	0.990 (1.049, 0.932)	1.014 (1.080, 0.949)	1.018 (1.091, 0.945)	0.927

HR, heart rate; IBI, interbeat interval; SDNN, standard deviation of all NN intervals; SDANN, standard deviation of the averaged NN intervals, calculated from 5 min epochs; RMSSD, the square root of the mean of the sum of squares of differences between adjacent NN intervals; pNN50, percentage of adjacent NN intervals that differ by greater than 50 ms; Ti, triangular index; HF, high frequency power; LF, low frequency power; LF:HF, ratio of LF to HF power; VLF, very low frequency power; SD1:SD2, the ratio of the SD of beat-to-beat IBI variability (SD1) against the SD of long-term IBI variability (SD2). Baseline results expressed as means (95% CI) and average on treatment represents the average of change at 6 and 12 months following each treatment based on estimated marginal means (adjusted for age, sex, ethnicity, BMI and baseline values), except [a] geometric means (95% CI) * Treatment effect versus placebo (Average on treatment divided by change on placebo at 6 and 12 months).

3.2.3 Discussion

An increased LC *n*-3 PUFA consumption has been associated with higher HRV (105,107,204), suggesting that populations with low LC *n*-3 PUFA tissue status might be at greater risk of arrhythmic events or SCD. Increasing LC *n*-3 PUFA content in cardiomyocyte membranes in animals reduced intrinsic pacemaker rate by altering ion channel currents and reduced pacemaker current in sinoatrial node cells resulting in a decreased HR and increased HRV (9,84). In the day-time, beat to beat variation of the heart rate is constantly occurring as a response to the physiological demands arising from daily activities and events, and it would be expected that supplementation with LC *n*-3 PUFA would increase the variability of the shorter phase HRV parameters, such as RMSSD, pNN50 and HF. However, these parameters didn't significantly change after the fish oil supplementation. A potential explanation could be the fact that the population studied already had a good EPA and DHA tissue status so, although the intervention increased the proportions of EPA and DHA in erythrocyte membranes in a dose-dependent manner compared to placebo, it might not have added an additional benefit in increasing short-term HRV indices that may be more related to membrane properties of cardiac myocytes. The significant treatment effect observed in SDANN and in VLF power in the day-time analysis seems to derive from the group supplemented with 0.9 g with the other supplemented groups not presenting any significant changes, which makes these results likely to be a type I statistical error. The day-time analysis suggests that supplementation with LC *n*-3 PUFA did not have an effect on HRV. Since this population appeared to be, on average, replete in LC *n*-3 PUFA from the start of the intervention, with their mean omega-3 index (O3I) near the 8% cut-off suggested for optimal cardioprotection (205), the logical next step is to assess HRV in a healthy population with a low LC *n*-3 PUFA status compared to a population with an adequate LC *n*-3 PUFA status. For this purpose, a cross sectional study using vegans as a model of a LC *n*-3 PUFA deficient population (the OM3GA study) was planned and will be presented in the next chapter.

Chapter 4 A comparison of heart rate variability, *n*-3 PUFA status and lipid mediator profile in age- and BMI-matched middle-aged vegans and omnivores

4.1 Introduction

The major dietary source of LC *n*-3 PUFAs is seafood and they can also be found in lean red meat, in organ meat, such as liver, in eggs and in very small amounts in dairy products. Consequently, vegans who do not eat any animal-derived products have a diet naturally free from the LC *n*-3 PUFAs eicosapentaenoic acid (20 : 5 *n*-3, EPA) and docosahexaenoic acid (22 : 6 *n*-3, DHA) (159). The main *n*-3 PUFA in vegan diets is α-linolenic acid (18 : 3 *n*-3, ALA), found in plant foods such as flaxseed, walnut, soybean, hempseed, chia seed and respective oils (206).

Having a diet devoid of LC *n*-3 PUFA, vegans present blood lipid LC *n*-3 PUFA concentrations that are only a third of the level in omnivores (159). Although EPA and DHA can be endogenously synthesised from the downstream metabolism of ALA, the efficiency of this conversion is limited in humans (207). LC *n*-3 PUFA, especially DHA, is rapidly incorporated into a variety of cells, primarily into phospholipids of the plasma membrane, including the cardiac cells (cardiomyocytes). Dietary alterations of LC *n*-3 PUFA in cardiomyocyte membranes influence the function of membrane channels and receptors and, consequently, the cells' contractile function (14,208,209). LC *n*-3 PUFA can also be oxygenated into numerous bioactive lipid mediators which act as precursors of the SPMs actively involved in the resolution of inflammation (210).

It has been shown that LC *n*-3 PUFA are preferentially incorporated in cardiomyocytes and inhibit the synthesis of AA which may result in altered production of eicosanoids. The alteration in the type and amount of eicosanoids generated, which in turn will originate different lipid mediator profiles, has been suggested as an explanation for the anti-arrhythmic properties of LC *n*-3 PUFA via the effects of these changes on intercellular calcium release and Ca^{2+} channels (173). This may have further effects on the cardiac autonomic function of the heart and

we therefore hypothesise that this might be associated with lower HRV in vegans, as a result of lower LC *n*-3 PUFA status, compared to omnivores. Animal, epidemiological and clinical studies evaluating the effects of LC *n*-3 PUFA on CV health outcomes have been mainly positive (144). This poses a conundrum: vegan dietary patterns are associated with lower blood pressure, lipids and a lower body mass index (BMI), all important risk factors for CVD (211), yet vegans are depleted in LC *n*-3 PUFA fatty acids associated with a cardioprotective role. The Omega-3 index (O3I), which represents the EPA + DHA content of erythrocyte membranes (expressed as a percentage of total fatty acids), has been emerging as a risk factor for CVD (212).

4.2 Hypothesis

The primary hypothesis of the study is that vegans have higher HR/shorter IBI and lower HRV compared with omnivores.

4.3 Aims

This study aims to compare HRV between vegans and age/sex/BMI-matched omnivore controls, representing populations with low and high tissue LC *n*-3 PUFA status, respectively. The results of this study will provide cross-sectional data to determine the variability of the outcome measures in this population in order to calculate the sample size needed if there were enough evidence for a link to justify a RCT of LC *n*-3 PUFA supplementation.

4.4 Study design, participants and recruitment

4.4.1 Study design

This is an observational cross-sectional pilot study being conducted at KCL. Recruitment onto the study was completed in two phases: the first phase ran between May and August 2013 and the second phase ran between May and August 2014.

4.4.2 Ethics and consent

Ethical approval was obtained from KCL research ethics committee (BDM/12/13-84) and written informed consent was given by participants.

4.4.3 Participants

Inclusion criteria: Healthy non-smoking men and women aged 40-70 y who report being vegan (for at least 2 y) or follow a mixed diet including meat, fish, eggs and dairy-containing foods (omnivore). Vegan subjects were matched with omnivore controls for sex, age (±5 y) and BMI (±2 kg/m^2).

Exclusion criteria: Reported history of CVD (myocardial infarction, angina, venous thrombosis, stroke, atrial fibrillation, pacemaker)**,** type 1 diabetes mellitus or type 2 diabetes (fasting plasma glucose ≥ 7 mmol/L)**,** cancer (excluding basal cell carcinoma) in the past five years**,** chronic renal, liver or inflammatory bowel disease**,** history of drug or alcohol abuse (previous weekly alcohol intake >60 units/men or 50 units/women), current self-reported weekly alcohol intake exceeding 28 units**, c**urrent use of marine *n*-3 PUFA supplements, pregnancy**,** weight change of more than 3 kg in the previous 2 months**,** BMI <18.5 and >35 kg/m^2.

4.4.4 Outcome measures

The **primary independent variable** is erythrocyte EPA and DHA content as a % of total fatty acids. Total plasma fatty acid composition (which would reflect more recent dietary intake in healthy populations) will also be analysed for comparison between groups. The **primary dependent variables** are HR/IBI and time-domain parameters of different components of HRV: SDNN, the most commonly reported marker of HRV and an indication of overall HRV, mainly determined by day/night differences; and RMSSD, an indicator of beat-to-beat, respiration-driven variability representing parasympathetic cardiac regulation.

Secondary outcomes include other time- and frequency-domain and non-linear parameters of HRV, fasting blood lipid profile (total, low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol and triacylglycerol concentrations), total fatty acid composition of plasma and erythrocyte membrane, plasma oxygenated lipid mediator profile, vitamin B_{12}, fasting glucose concentrations, anthropometric measurements (weight, height, BMI, waist circumference, % body fat), HR, blood pressure, activity levels (in counts per

minute) and background diet (food frequency questionnaires) in order to compare risk factors for CVD in vegans and omnivores.

4.4.5 Recruitment methodology

Vegan participants were recruited through external advertising to the following vegan communities: the Vegan Society [3], London Vegans [4] and London Vegan Meetup [5]. Omnivore participants were recruited through internal and external email circulars and posters among KCL students, staff and participants from the CRESSIDA study. The study was also promoted via Facebook, Gumtree and Twitter, flyer distribution to vegan restaurants and shops selling vegan foods and supplements throughout London, and through the participation in various food events, including the Big IF event (Hyde Park, 8th June 2013), the London Vegan Potluck dinner (3rd July 2013 and 4th June 2014) and the Vegan Drinks (15th May 2014). Volunteers who responded to advertisements were given more information about the study (**Appendix 2**), completed a recruitment questionnaire via telephone call or e-mail to establish their eligibility for the study and were sent a study information sheet. The eligible participants that were willing to participate attended a morning clinic visit at the MRU at Franklin-Wilkins building (King's College London, Waterloo campus) booked according to participant's availability. Recruitment methodology is summarised in **Figure 15**.

[3] The Vegan Society, 47 Highlands Road, Leatherhead, Surrey.
[4] London Vegan Office, 7a Deansbrook Road, Edgware, Middlesex, HA8 9BE.
[5] www.meetup.com/londonvegan/

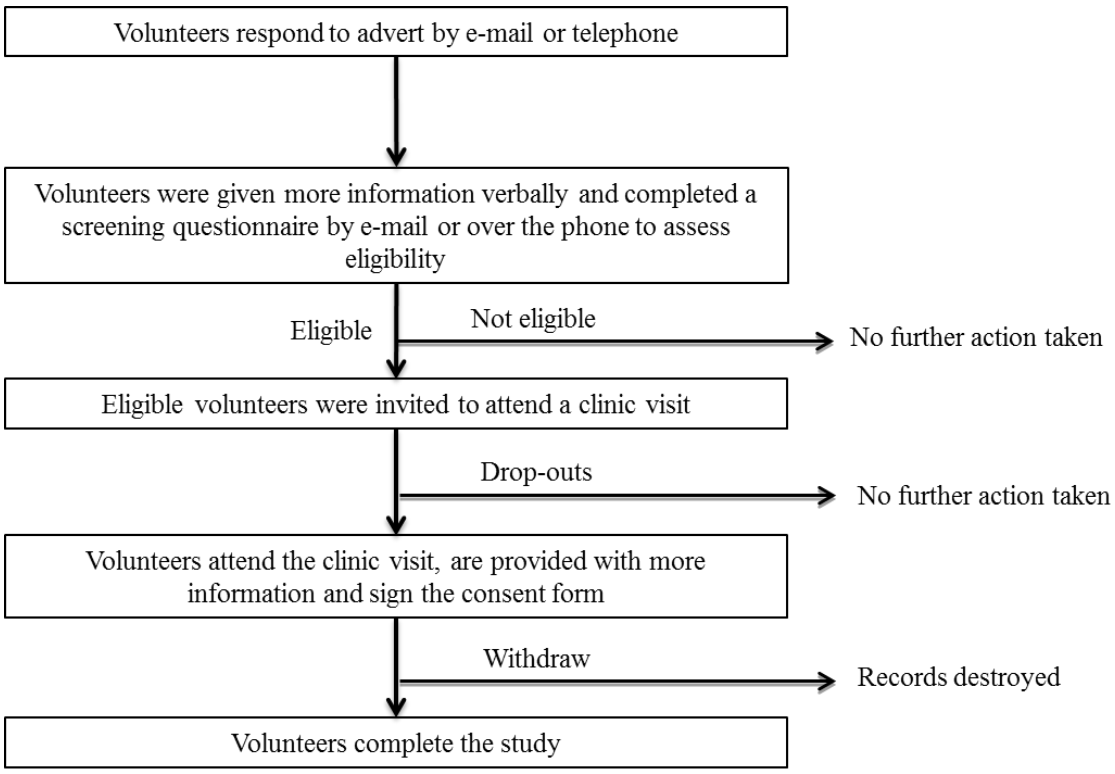


Figure 15 – Diagram showing volunteer recruitment.

4.5 Study days

4.5.1 Study protocol

The clinic visits took place in the morning (between 8.00 and 11.00 am) and lasted approximately 1 hour. Volunteers were instructed to fast for 12 h before the clinic visit and consume nothing but water until attending the clinic. On arrival, the study protocol was explained in detail and participants were given the opportunity to ask questions before signing the consent form. After a 10 minute rest, three measurements of seated blood pressure were taken using an A&D Medical UA-767Plus auto upper arm blood pressure monitor (A&D Instruments Limited, San Jose, USA), in accordance with guidelines from the BHS. Height, body weight and percentage body fat, and waist circumference were measured using a stadiometer, a Tanita weighing scale and a tape measure, respectively. Participants also completed a food frequency questionnaire (FFQ) that was checked for completeness and any missing data was verified directly with the participant. A fasting blood sample (20 ml equivalent to 4 tsp) was taken to check that lipids, haematology, liver function and glucose were within specified limits and a sample was set aside for plasma fatty acid and erythrocyte lipid analyses.

Volunteers were fitted with the Actiheart monitor and asked to wear it for 24 h. A diary was provided (**Appendix 3**) during the recording period to keep a register of all the daily activities (activities/exercise, meals or naps). After 24 h the devices were returned by courier. Participants were offered a light breakfast after completing the study visit. Screening blood samples were sent via courier to King's College Hospital, Denmark Hill for analysis. Participants were provided with the results of the blood screening analysis and a letter to their general practitioner upon request detailing results and drawing attention if necessary to any out of range values.

4.5.2 Anthropometric measurements

Height was measured using a stadiometer to the nearest 1 cm while participants were barefoot, standing upright with heels and shoulders against the measuring rod and looking straight ahead. Waist circumference was measured using a metric measuring tape to the nearest 0.1 cm in the standing position; waist circumference was measured at the midway point between the iliac crest and the lowest rib with the participant's waist uncovered or with very light clothing. Readings were taken at the end of a normal exhalation. Weight was measured to the nearest 0.1 kg and percentage body fat was assessed by bioelectrical impedance analysis using a TANITA BC-418 segmental body composition analyser.

4.5.3 Dietary assessment

The EPIC-Norfolk FFQ (pages 2 to 10, **Appendix 4**) was used in this study to assess patients' background diet over the past 12 months as well as to verify self-classification of dietary status of eligible volunteers. The FFQ consists of two parts: the first part asks about the frequency of consumption of 130 foods. Participants are asked to choose from nine frequency categories ranging from "never or less than once/month" to "6+ times per day". The second part consists of additional questions to provide information about extra foods consumed frequently (more than once per week) that were not mentioned in the first part, type and amount of milk, breakfast cereals, cooking methods, type of fats used and supplements regularly taken. Data obtained from the completed FFQ was entered into an in-house customised Microsoft Access database and exported to Microsoft Excel for further processing. The daily nutrient intakes were estimated using food codes and portion size calculations. The average portion size for each

response was determined using a sliding scale of fractions of portions corresponding to those used in the EPIC study. The specific macronutrient, micronutrient and individual LC *n*-3 PUFA intake calculations were determined using the nutrient composition values from the McCance and Widdowson's 'The Composition of Foods Integrated Dataset' [6] with additional data obtained from manufacturers and USDA values when required.

4.5.4 Heart Rate Variability

Heart rate variability was measured using the Actiheart monitoring equipment (CamNtech Ltd, Cambridge, UK). This is a small, light-weight (<10 g) waterproof device that records acceleration (counts per minute, cpm) as a measure of physical activity, HR, and all inter-beat intervals for approximately 24 h. Skin preparation took part including shaving of chest hair, use of alcohol wipes to clean and dry the skin and use of an abrasive pad (Unilect™) to remove the top layer of skin cells. Two ECG electodes (SP-50, 50 mm round, Pulse Medical) were placed on the chest to fit the Actiheart monitor. A short signal test, involving around a 5 minute walk with the participant, was performed before programming for the 24 h recording to confirm that the level of R wave signal being picked up by the Actiheart was adequate. Data processing of the 24 h HRV recordings was carried out using the Actiheart software (version 4.0.91, CamNtech Ltd, Cambridge, UK) and Kubios HRV analysis software (Biosignal Analysis and Medical Imaging Group, Department of Physics, University of Kuopio, Finland). For more detailed information please refer to the general methods chapter (**Chapter 2**). HRV, HR/IBI and accelerometry data were analysed for the full length of recording time (minimum of 18 h, up to 24 h). Further analysis was carried on a standardised day-time period of 8h and sleep-time period of 2 h to remove the influence of variability in recording duration on HRV parameters. The 8 h and 2 h time periods were selected as the first 8 h and 2 h of good quality data, respectively, from the start of day-time and sleep-time periods (excluding any noisy sections where signal was poor, and periods of awake time at night assessed by and increased heart rate matched with increased accelometry data).

[6]http://tna.europarchive.org/20110116113217/http://www.food.gov.uk/science/dietarysurveys/dietsurveys/

4.5.5 Blood sample collection, handling and analysis

Blood was drawn into vacutainer tubes. Blood for plasma glucose analysis was collected into 4 ml fluoride oxalate tubes. Blood for plasma lipids (triacylglycerol, total cholesterol, LDL- and HDL-cholesterol), liver function, vitamin B_{12} and 25-hydroxy vitamin D analysis was collected into 5 ml SST^{TM} serum tubes containing a gel for separation. Blood for the full blood count and IL-6 analysis was collected into 4 ml EDTA tubes. These bloods were sent by courier and analyses were carried out at the department of clinical biochemistry at King's College Hospital (ViaPath). Glucose and lipids were analysed following enzymatic methods using reagents supplied by Bayer Diagnostics Europe Ltd (Bayer House) using an ADVIA 2400 analyser (Siemans Healthcare Diagnostics); IL-6 was analysed using a high-sensitivity cytokine chip array assay (Human cytokine HS X biochip; Randox Laboratories Limited); and Serum vitamin D and B_{12} concentrations were analysed using the ADVIA Centaur total vitamin D and vitamin B_{12} immunoassays (Siemens Healthcare Diagnostics Ltd). Blood for the erythrocyte membrane phospholipid fatty acid and plasma fatty acid composition was collected into 6 ml EDTA tubes and analysed at the diabetes and nutritional sciences department at King's College London. Further details about the blood handling protocol can be found in **Appendix 5**.

Following the blood collection, the 6ml EDTA tubes were centrifuged at 1500 g for 15 minutes at 4 ºC. Plasma was aliquoted into cryovials and stored at -80 ºC until analysed. The remaining haematocrit was washed with isotonic saline 3 times to remove residual plasma and the buffy coat (white blood cells). A 0.5 ml aliquot of the washed cells was lysed with equivalent volume of deionised water. Following the erythrocyte lyses, 5.5 ml of isopropanol (containing butylated hydroxyl toluene, added as an antioxidant in the concentration of 50 mg/L) was added to denature the proteinatious component of the cell membrane. This was followed by the addition of 3.5 ml chloroform to extract the lipids. The solution was vortexed and centrifuged to pelletize the cellular debris. The supernatant was then transferred to a glass tube with a cap and stored at -20 ºC until analysed.

4.5.6 Determination of the fatty acid composition of plasma and erythrocyte membrane lipids

Plasma fatty acid composition was determined based on Lepage and Roy 1988 method, substituting toluene for benzene and using pentadecanoic acid as an internal standard. Erythrocyte membrane phospholipid fatty acid composition was determined as previously described (192). Proportions of fatty acids were expressed as weight % (Refer to chapter 2 – Methods, section 2.3, for further details).

4.5.7 Extraction and determination of lipid mediators in plasma

Lipid mediators are produced from the oxidation of PUFA through enzymatic or non-enzymatic free radical mediated reactions. The three major enzymatic pathways are catalysed by COX, LOX and CYP monooxygenases. These lipidomic analyses were performed as a collaborative work with the laboratory of Prof. Anna Nicolaou at the University of Manchester. In this study the lipid mediators were extracted from plasma and analysed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS). This assay targets prostanoids and hydroxyl fatty acids derived from the main PUFA, including dihomo-gamma-linolenic acid (DGLA), LA, ALA, AA, EPA and DHA. The method has been previously described (210) Briefly, an ice-cold methanol solution (15% v/v) was added to the plasma samples to help extracting the lipid mediators and facilitate the denaturation and precipitation of proteins. After adding two deuterated internal standards to each sample (PGB2-*d*4 for prostanoids and 12-HETE-*d*8 for hydroxyl fatty acids), the samples were centrifuged and any precipitate was removed. The clear supernatant was then acidified to pH 3 using a HCL solution (0.1 M). The acidified extract was then loaded onto a preconditioned solid phase extraction cartridge and washed three times, firstly with methanol solution (15% v/v), followed by deionised water and lastly hexane. Finally, the compounds of interest are eluted in methyl formate. The solvent is evaporated under nitrogen and the lipid residue is resuspended in a small volume of ethanol (70% v/v). After centrifuging, the samples were transferred to amber sample vials and stores at -20 ºC for LC/ESI-MS/MS analysis with the use of an Acquity UPLC system coupled to an electrospray triple quadrupole Xevo TQ-S mass spectrometer (Water, UK).

Calibration lines for all compounds of interest were generated during the assay using a cocktail of commercially-available lipid standards (Cayman) to accurately quantify each compound in plasma. Prostanoids and hydroxyl fatty acids were analysed through two separate LC/ESI-MS/MS runs using gradient methods. Each sample was analysed twice. The chromatographic analysis was performed on a C18 column (ACQUITY UPLC BEH, 1.7 µm, 2.1 mm x 50 mm) and the analytes were separated at a flow rate of 0.6 ml/min.

4.6 Statistical analysis

A sample size of twenty-three in each group has 80% power to detect a difference between SDNN means of 25 ms and between RMSSD means of 15 ms with a significance level of 0.05 (two-tailed), based on SD of 30 and 18 ms, respectively, obtained from sleep-time HRV recordings in a previous cohort of middle-aged to older healthy men and women (186).

Statistical analyses were performed using IBM SPSS Statistics 21.0 (Statistical Product and Service Solutions, IBM Corp). Chi-squared (χ^2) tests for categorical variables and independent samples t-test for continuous variables were used to assess the differences between vegan and omnivore subjects' characteristics, dietary intakes, erythrocyte and plasma fatty acids, and lipid mediators. Data normality was determined using Shapiro-Wilk test and by visual inspection of the data distribution on the histogram and Q-Q plots. Non-normally distributed data were normalised by natural logarithm (LN) (results shown as geometric means and 95% CI) before analysis by independent *t* test. If data was not normalised by LN transformation, a Mann–Whitney *U* test was applied to compare groups (results shown as medians with lower and upper quartiles). In the case of lipid mediators, results from non-normally distributed data analysed by Mann–Whitney *U* test were shown as medians with minimum and maximum values due to the proportion of undetectable concentrations of LC *n*-3 PUFA-derived mediators in omnivores and vegans. For HRV analysis, normally distributed raw data or LN transformed data were analysed by univariate ANCOVA, adjusted for sex, age, BMI and, in the case of day-time and 24 h data, for physical activity (accelerometry data). Results are expressed as estimated marginal means (95% CI), adjusted for sex, age, BMI and 24 h activity for 24 h HRV and sleep-time – day-time HRV, or sex, age, BMI and 8 h activity for 8 h day-

time HRV, or adjusted for sex, age and BMI only for 2 h sleep-time. Estimated marginal means and 95% CI from data that were LN transformed before analysis by ANCOVA were back-transformed and expressed as geometric means and 95% CI. Data that could not be normalised by LN transformation were analysed using Mann–Whitney *U* test and significance values are presented unadjusted, with results shown as medians (lower and upper quartiles).

4.7 Results

4.7.1 Subjects

From the 127 subjects that responded to the adverts and completed the screening questionnaire, 62 were eligible and invited to take part in the study. Of these 49 attended the clinic visit and 47 completed the study (**Figure 16**).

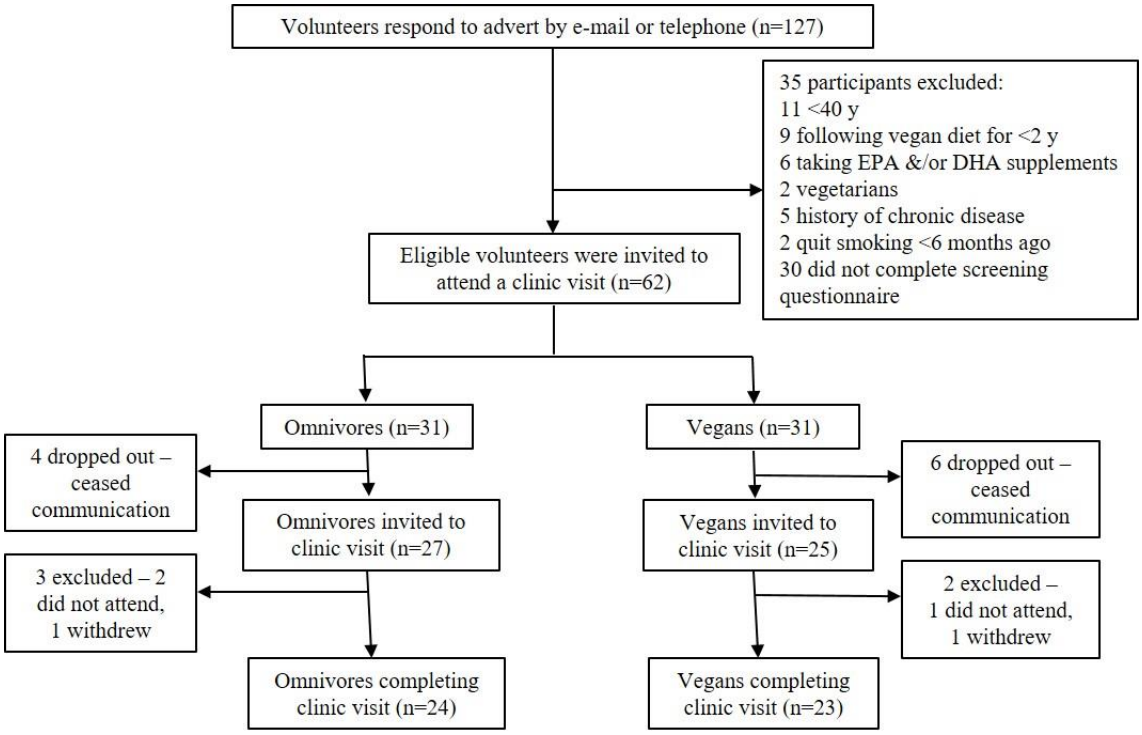


Figure 16 - Study consort diagram.

4.7.2 Characteristics of study subjects

The characteristics of the study subjects are presented in **table 10**. The study group was formed of a total of 23 vegan subjects (8 male, 15 female) with a mean age of 49 ± 8 y and a mean BMI of 23.5 ± 4.4 kg/m^2 and 24 omnivores (12 male, 12 females) with a mean age of 54 ± 9 y and a mean BMI of 23.3 ± 2.8 kg/m^2. There were no significant differences in age or BMI,

or distribution of sex between groups, although the sex distributions were not fully balanced across groups. Furthermore, there were no significant differences in waist circumference and % body fat. Seated resting SBP was significantly lower in the vegan subjects while the seated resting HR was significantly lower in the omnivore group. There was no significant difference in DBP between groups. Biochemistry results for the vegan and omnivore subjects are reported in **Table 11**. Both fasting serum total and LDL-cholesterol were significantly lower in vegans, but there were no differences in fasting plasma glucose, serum triacylglycerol, serum HDL-cholesterol, serum vitamin B_{12}, serum 25-hydroxy vitamin D or IL-6 concentrations, nor blood haemoglobin concentrations, indicating that the vegan group did not differ in vitamin D status and were likely to be taking dietary vitamin D (65% taking supplements) and B_{12} supplements (61% taking supplements).

Table 10 – Characteristics of subjects who completed the study (n=47).

	Omnivore (n=24)	Vegan (n=23)	*P* value[†]
Sex			
Male	12 (50)	8 (35)	0.292[§]
Female	12 (50)	15 (65)	
Ethnicity			
White	21 (88)	21 (91)	0.289[§]
Black African/Caribbean	0 (0)	1 (5)	
South Asian	2 (8)	0 (0)	
Other	1 (4)	1 (5)	
Vitamin B_{12} supplement use	8 (33)	14 (61)	0.059[§]
Vitamin D supplement use	5 (21)	15 (65)	0.003[§]
Age (years)	54 ± 9.1	49 ± 7.9	0.081
BMI (kg/m^2)	23.3 ± 2.8	23.5 ± 4.4	0.896
Waist circumference (cm)			
Male	91.7 ± 6.5	97.2 ± 5.9	0.165
Female	88.8 ± 12.5	86.0 ± 14.8	0.617
% body fat			
Male	19.3 ± 7.9	21.1 ± 5.9	0.572
Female	30.6 ± 7.7	29.4 ± 9.9	0.751
SBP (mmHg)	118 ± 9.8	111 ± 9.9	**0.032**
DBP (mmHg)	75 ± 9.3	74 ± 8.2	0.703
Heart Rate (bpm)	63 ± 10.8	70 ± 9.0	**0.017**

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; bpm, beats per minute.
Results are expressed as number of subjects (%) or mean ± SD. [†] Using independent samples *t*-test, except [§] χ^2 test.

Table 11 – Biochemistry results of subjects who completed the study (n=47).

	Omnivore (n=24)	Vegan (n=23)	P value†
Plasma fasting glucose (mmol/L)	5.2 ± 0.43	5.1 ± 0.40	0.616
Serum triacylglycerol (mmol/L) [a]	0.77 (0.66, 0.92)	0.76 (0.64, 0.90)	0.849
Serum total cholesterol (mmol/L)	4.9 ± 0.86	4.1 ± 0.66	**0.001**
Serum LDL-C (mmol/L) [a]	2.81 (2.49, 3.17)	2.16 (1.94, 2.41)	**0.002**
Serum HDL-C (mmol/L)			
Male	1.52 ± 0.35	1.29 ± 0.23	0.118
Female	1.71 ± 0.24	1.66 ± 0.42	0.725
Haemoglobin (g/L)			
Male	14.4 ± 0.6	14.4 ± 0.8	0.805
Female	13.2 ± 1.0	13.5 ± 1.0	0.460
Serum vitamin B_{12} (ng/L)	442 ± 216	358 ± 117	0.108
Serum 25-hydroxy vitamin D (nmol/L)	54.3 ± 20.9 **	55.6 ± 26.5	0.854
Interleukin-6 (ng/L) [b]	0.93 (0.25, 1.54) **	1.07 (0.23, 3.05)	0.701‖

HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol
Results are expressed as mean ± SD, [a] geometric mean (95% confidence intervals) or [b] median (upper and lower quartiles). † Using independent samples *t*-test, except ‖Mann-Whitney U test ** n=23 due to sample loss.

4.7.3 Dietary intake

Dietary intakes (excluding supplements) are shown in **table 12**. Vegans and omnivores had comparable energy (kcal), total fat (%E) and monounsaturated fatty acids (MUFA, %E) intakes. Omnivores had significantly higher protein (%E), saturated fatty acids (SFA, %E) and food-derived vitamin B_{12} (µg) intakes and vegans had significantly higher carbohydrate (%E), PUFA (%E) and LA (g) intakes. As expected, vegans had no dietary intake of EPA and DHA, hence omnivores obtained significantly higher intake of these fatty acids. Dietary intake of vitamin B12 was below the lower reference nutrient intake (LRNI) in 30.4% of the vegan subjects. All omnivore subjects met the reference nutrient intake (RNI) of 1.5µg/day of vitamin B_{12} (through food intake) compared to 56.5% of vegan subjects.

Table 12 – Mean daily dietary intakes of vegans and omnivores assessed by food frequency questionnaire.

	Omnivore (n=24)	**Vegan (n=23)**	***P value*** [†]
Energy (kcal)	1953 ± 478	1833 ± 662	0.477
Protein (%E)	16.6 ± 2.2	13.3 ± 2.4	**< 0.001**
Carbohydrates (%E)	49.2 ± 7.3	56.5 ± 11.6	**0.013**
Total fat (%E)	33.8 ± 5.9	30.9 ± 9.5	0.216
SFA (%E)	11.8 ± 2.6	6.3 ± 1.7	**< 0.001**
MUFA (%E) [b]	13.5 (12.1, 15.0)	11.6 (9.3, 14.4)	0.136 ‖
PUFA (%E)	6.0 ± 1.2	9.6 ± 3.1	**< 0.001**
18 : 2*n*-6, LA (g) [b]	7.6 (5.9, 10.2)	10.5 (7.3, 18.5)	**0.025** ‖
18 : 3*n*-3, ALA (g) [b]	0.7 (0.5, 1.0)	0.8 (0.5, 1.2)	0.425 ‖
20 : 5*n*-3, EPA (g) [b]	0.14 (0.09, 0.24)	0.00 (0.00, 0.00)	**< 0.001** ‖
22 : 6*n*-3, DHA (g) [b]	0.45 (0.30, 0.81)	0.00 (0.00, 0.00)	**< 0.001** ‖
Vitamin B12 (µg)	6.9 ± 2.7	2.3 ± 1.9	**< 0.001**

ALA, α-linolenic acid; DHA, docosahexaenoic acid; %E, percentage energy; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.
Results are expressed as mean ± SD, except [b] median (upper and lower quartiles). [†] Using independent samples *t*-test, except ‖ Using Mann-Whitney U test.

4.7.4 Plasma and erythrocyte lipid fatty acid composition

Plasma and erythrocyte membrane fatty acid composition are presented in **Table 13**. Vegans had a significantly higher proportion of plasma and erythrocyte LA and adrenic fatty acid (a metabolite of AA), plasma ALA and erythrocyte DGLA, compared to omnivores. Both plasma and erythrocyte membrane proportions of EPA and DHA, plasma palmitic acid, and erythrocyte docosapentaenoic acid (DPA) *n*-3 were significantly lower in vegans compared to omnivores. Vegans had a significantly lower O3I, with a geometric mean of 2.7% compared to 5.4% in omnivores, although both groups would be considered below the proposed O3I cut-off of > 8% for optimal cardiovascular protection (205). Erythrocyte LA : ALA ratios were inversely moderately correlated with erythrocyte EPA contents in vegans (r = -0.541, P = 0.008, n = 23), but not DPA *n*-3 or DHA contents; with no significant correlations in the erythrocyte lipids of omnivores. In plasma, the ratio of LA : ALA was inversely moderately correlated with plasma DPA *n*-3 (r = -0. 576, P = 0.004, n = 23) and DHA (r = -0.498, P = 0.016, n = 23) in vegans and plasma DPA *n*-3 only in omnivores (r = -0.474, P = 0.019, n = 24).

Table 13 - Plasma and erythrocyte membrane fatty acid composition (weight %) of participants (n=47).

Fatty acid	**Omnivore (n=24)**	**Vegan (n=23)**	**Mean Δ**	**(95% CI)**	***P value*** [†]
Plasma					
16 : 0 (Palmitic)	20.8 (20.4, 21.3)	19.3 (18.6, 20.0)	**-1.49**	**(-2.31, -0.67)**	**0.001**
16 : 1*n*-7 (Palmitoleic)	1.79 (1.48, 2.10)	1.11 (0.91, 1.32)	**-0.67**	**(-1.04, -0.31)**	**0.001**
18 : 0 (Stearic)	7.62 (7.30, 7.94)	7.60 (7.15, 8.04)	-0.02	(-0.55, 0.50)	0.931
18 : 1*n*-9 (Oleic)	18.5 (17.6, 19.3)	18.9 (17.9, 19.8)	0.37	(-0.89, 1.62)	0.559
18 : 2*n*-6 (LA)	27.1 (26.0, 28.2)	33.1 (31.9, 34.4)	**6.06**	**(4.43, 7.68)**	**< 0.001**
18 : 3*n-3* (ALA) [a]	0.53 (0.48, 0.59)	0.71 (0.59, 0.85)	**1.34***	**(1.09, 1.64)**	**0.006**
20 : 3*n*-6 (DGLA)	1.42 (1.28, 1.55)	1.42 (1.28, 1.57)	0.01	(-0.18, 0.20)	0.952
20 : 4*n*-6 (AA)	6.68 (6.12, 7.25)	6.55 (5.94, 7.16)	-0.13	(-0.94, 0.68)	0.745
20 : 5*n-3* (EPA) [a]	1.03 (0.79, 1.34)	0.47 (0.40, 0.55)	**0.46***	**(0.34, 0.62)**	**< 0.001**
22 : 4*n*-6 (Adrenic) [a]	0.20 (0.19, 0.21)	0.23 (0.21, 0.25)	**1.14***	**(1.01, 1.28)**	**0.036**
22 : 5*n*-6 (DPA *n*-6)	0.26 (0.21, 0.30)	0.20 (0.15, 0.26)	-0.05	(-0.12, 0.02)	0.146
22 : 5 *n-3* (DPA *n-3*)	0.59 (0.53, 0.64)	0.51 (0.44, 0.59)	-0.07	(-0.16, 0.02)	0.113
22 : 6 *n-3* (DHA) [a]	2.23 (1.94, 2.57)	0.91 (0.80, 1.05)	**0.41***	**(0.34, 0.49)**	**< 0.001**
Erythrocyte membrane					
16 : 0 (Palmitic)	16.8 (15.4, 18.2)	17.6 (16.7, 18.6)	0.81	(-0.87, 2.48)	0.337
16 : 1*n*-7 (Palmitoleic)[b]	0.41 (0.30, 1.57)	0.31 (0.21, 0.50)	-	-	**0.016** [‖]
18 : 0 (Stearic) [a]	15.6 (14.9, 16.3)	16.3 (15.6, 17.1)	1.05*	(0.99, 1.14)	0.135

18 : 1 *n*-9 (Oleic)	15.7 (15.1, 16.3)	15.4 (14.7, 16.2)	-0.23	(-1.15, 0.68)	0.609
18 : 2 *n*-6 (LA)	11.7 (11.0, 12.3)	13.3 (12.5, 14.1)	**1.64**	**(0.64, 2.64)**	**0.002**
18 : 3 *n-3* (ALA) [a]	0.34 (0.26, 0.45)	0.32 (0.27, 0.38)	0.92*	(0.67, 1.27)	0.610
20 : 3 *n*-6 (DGLA) [a]	1.78 (1.64, 1.94)	2.02 (1.84, 2.22)	**1.13***	**(1.01, 1.28)**	**0.042**
20 : 4 *n*-6 (AA)	15.9 (14.9, 16.9)	15.6 (14.4, 16.9)	-0.27	(-1.82, 1.27)	0.725
20 : 5 *n-3* (EPA)	1.26 (1.07, 1.45)	0.67 (0.52, 0.81)	**-0.59**	**(-0.83, -0.36)**	**< 0.001**
22 : 4 *n*-6 (Adrenic)	2.75 (2.47, 3.03)	3.83 (3.50, 4.16)	**1.08**	**(0.66, 1.50)**	**< 0.001**
22 : 5 *n*-6 (DPA *n*-6)	0.38 (0.27, 0.49)	0.52 (0.40, 0.64)	0.14	(-0.02, 0.30)	0.078
22 : 5 *n-3* (DPA *n-3*)	2.62 (2.36, 2.88)	2.15 (1.94, 2.36)	**-0.47**	**(-0.80, -0.15)**	**0.005**
22 : 6 *n-3* (DHA) [a]	4.19 (3.63, 4.83)	2.07 (1.85, 2.32)	**0.49***	**(0.41, 0.59)**	**< 0.001**
Omega 3 index [a]	5.42 (4.73, 6.20)	2.71 (2.40, 3.05)	**0.50***	**(0.42, 0.60)**	**0.006**

AA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; DPA, docasapentaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid. Results are expressed as mean (95% CI), except [a] Geometric mean (95% CI), [b] median (lower and upper quartile) [†] Using independent samples *t*-test, except [‖] using Mann-Whitney *U* test. *Exponents of mean differences in LN values (the ratio of the geometric mean in vegans:that in omnivores, with 95% CI of the geometric mean ratios). Total plasma fatty acid concentrations were (geometric means with 95% CI): omnivores (1869 mg/L, 1660, 2104; n=24), vegans (1998 mg/L, 1755, 2274; n=23); there were no significant differences between groups.

4.7.5 Lipid mediators in plasma

Figure 17 and **Figure 18** show all the lipid mediators included in the analysis protocol, including those that were not detected in the plasma of this study population. **Figure 17** presents the schematic outline of lipid mediators that derive from the *n*-6 PUFA fatty acids LA, DGLA and AA and **Figure 18** presents the schematic outline of lipid mediators that derive from the *n*-3 PUFA fatty acids ALA, EPA and DHA. **Table 14** shows *n*-3 and *n*-6 PUFA-derived lipid mediators detected in the fasting plasma of the two study groups. Overall, the lipid mediators derived from *n*-6 PUFA (mainly DGLA and LA), and plant derived *n*-3 PUFA (ALA) were higher in vegans compared to omnivores, and the lipid mediators derived from EPA and DHA were lower in vegans compared to omnivores, showing a clear difference in the lipidomic profile between the groups. SPMs (resolvins, protectins and maresins) were not detectable in the fasting plasma samples. Notably, in vegans there were markedly lower fasting plasma concentrations of 18-hydroxyeicosapentaenoic acid (18-HEPE), an EPA-derived precursor marker for resolvin E1 (RvE1), and undetectable concentrations of 17-hydroxydocosahexaenoic acid (HDHA), a DHA-derived precursor marker for resolvin D1, resolvin D2, and PDX, an isomer of protectin D1. 14-HDHA, another mediator arising from DHA and a precursor marker for the macrophage-derived maresin 1, was also much lower in vegan fasting plasma compared to omnivores. In summary, these data show that vegans have increased blood concentrations of oxygenated metabolites of LA and ALA compared to omnivores, and very low or undetectable concentrations of LC *n*-3 PUFA-derived oxygenated metabolites.

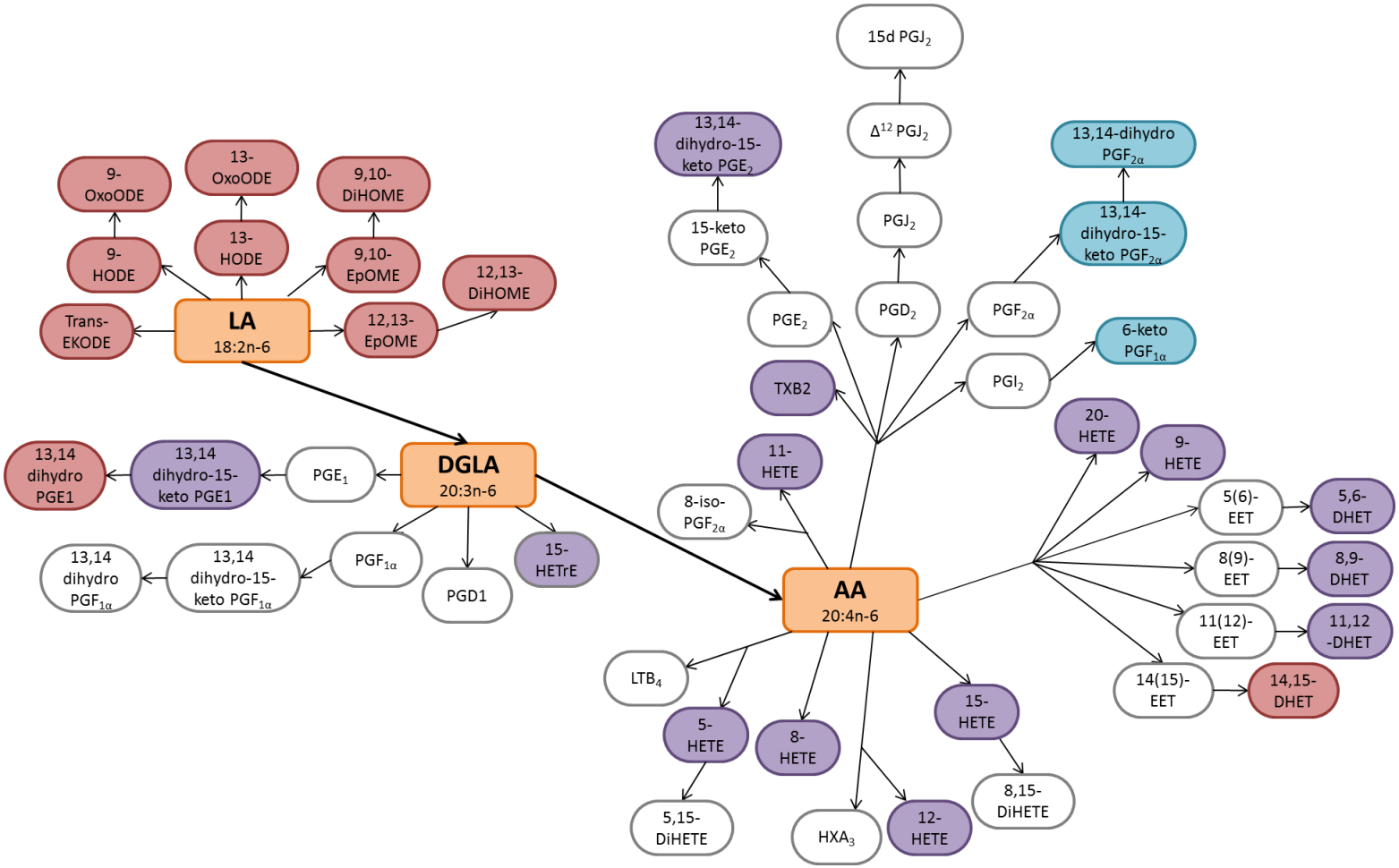


Figure 17 – Schematic outline of *n*-6 PUFA-derived lipid mediators analysed in blood plasma of study participants. Red = higher in vegans, blue = higher in omnivores, purple = no difference, white = not detected or below the limit of detection. OxoODE, oxooctadecadienoic acid; HODE, hydroxyoctadecadienoic acid; EpOME, epoxyoctadecenoic acid; DiHOME, dihydroxyoctadecenoic acid; EKODE, epoxyketooctadecenoic acid; TX, thromboxane; HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatetraenoic acid; DHET, dihydroeicosatetraenoic acid; HETrE, hydroxyeicosatrienoic acid; DiHETE,dihydroxyeicosatetraenoic acid; LT, leukotriene; HX, hepoxilin.

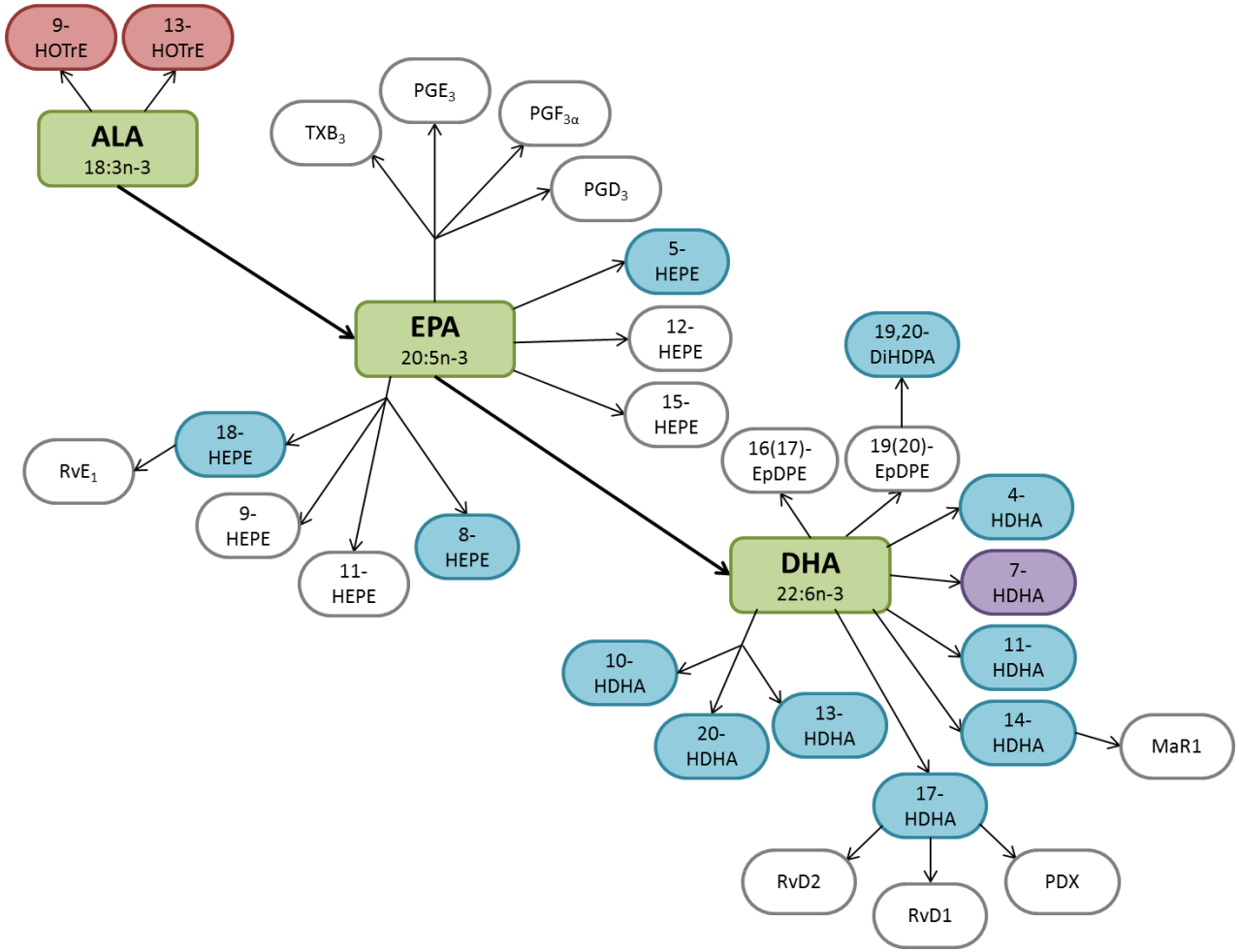


Figure 18 – Schematic outline of *n*-3 PUFA-derived lipid mediators analysed in blood plasma of study participants. Red = higher in vegans, blue = higher in omnivores, purple = no difference, white = not detected or below the limit of detection. HOTrE, hydroxyoctadecatrienoic acid; TX, thromboxane; PG, prostaglandin; HEPE, hydroxyeicosapentaenoic acid; Rv, resolvin; HDHA, hydroxydocosahexaenoic acid; PD, protectin D; MaR, maresin; EpDPE, epoxydocosapentaenoic acid; DiHDPA,dihydroxydocosapentaenoic acid.

Table 14 – Plasma concentrations of *n*-3 and *n*-6 PUFA-derived lipid mediators in the study participants (n=47).

Compound (ng/ml)	**Omnivore (n=24)**		**Vegan (n=23)**		***p value*** [†]
***n*-6 PUFA-derived**					
DGLA-derived					
15-HETrE	51.82	(36.94, 66.70)	53.87	(39.78, 67.96)	0.533
13,14-dihydro-15-keto PGE_1 [a]	17.45	(9.52, 31.99)	13.93	(7.82, 24.78)	0.519
13,14-dihydro PGE_1 [b]	0.000	(0.000, 4.332)	0.000	(0.000, 76.928)	0.001‖
LA-derived					
9-HODE [a]	2433	(1982, 2988)	5045	(4067, 6260)	<0.001
9 OxoODE [a]	477	(398, 571)	994	(771, 1282)	<0.001
13-HODE [a]	3320	(2717, 4056)	6536	(5483, 7791)	<0.001
13 OxoODE	335	(291, 379)	537	(467, 606)	<0.001
12(13) EpOME	389	(326, 451)	769	(622, 917)	<0.001
12,13-DiHOME	2820	(2159, 3480)	5544	(4527, 6561)	<0.001
9,10-DiHOME [a]	3.20	(2.48, 4.12)	7.40	(5.91, 9.27)	<0.001
9(10) EpOME [a]	258	(220, 303)	426	(351, 518)	<0.001
Trans EKODE [a]	300	(248, 362)	560	(425, 738)	<0.001
AA-derived					
6-keto $PGF_{1\alpha}$ [b]	8.20	(0.000, 59.14)	0.000	(0.000, 4.49)	<0.001‖
13,14-dihydro $PGF_{2\alpha}$ [a]	48.4	(34.57, 67.91)	20.0	(14.82, 26.97)	<0.001
13,14-dihydro-15-keto $PGF_{2\alpha}$ [b]	0.000	(0.000, 25.56)	0.000	(0.000, 12.79)	0.016 ‖
13,14-dihydro-15-keto PGE2 [a]	3.67	(2.584, 5.217)	3.07	(2.172, 4.347)	0.459
TXB2 [a]	10.3	(7.57, 14.14)	10.5	(6.08, 18.05)	0.968
5-HETE [a]	124	(96.4, 160.4)	132	(103, 168)	0.738
8-HETE	70.2	(56.77, 83.57)	74.5	(62.33, 86.60)	0.626
9-HETE [b]	12.82	(0.00, 70.99)	0.000	(0.000, 70.99)	0.478 ‖
11-HETE	62.3	(49.55, 75.13)	59.7	(49.68, 69.64)	0.736
12-HETE [b]	146	(96.8, 1214.4)	144	(82.2, 1625.8)	0.580 ‖
15-HETE	174	(145, 203)	186	(158, 214)	0.533
20-HETE [a]	284	(232, 348)	290	(229, 367)	0.896
5,6-DHET [a]	55.4	(47.09, 65.19)	52.0	(41.29, 65.37)	0.635
8,9-DHET [a]	71.6	(62.75, 81.81)	82.1	(67.40, 99.95)	0.238
11,12-DHET	204	(173, 235)	233	(207, 260)	0.147
14,15-DHET	260	(231, 290)	312	(272, 352)	0.036

Compound (ng/ml)	Omnivore (n=24)		Vegan (n=23)		*p* value[†]
***n*-3 PUFA-derived**					
ALA-derived					
9 HOTrE [a]	139	(111, 173)	206	(170, 250)	0.007
13 HOTrE [a]	150	(114, 198)	245	(201, 299)	0.005
EPA-derived					
5-HEPE [b]	75.7	(0.000, 462.2)	21.4	(0.000, 107.5)	<0.001 ‖
8-HEPE [b]	15.2	(0.000, 219.0)	0.000	(0.000, 21.0)	<0.001‖
18-HEPE [b]	74.8	(0.000, 624.8)	0.000	(0.000, 150.2)	<0.001 ‖
DHA-derived					
4-HDHA [b]	75.8	(38.18, 413.41)	45.7	(0.000, 102.33)	<0.001 ‖
7-HDHA [b]	0.000	(0.000, 202.4)	0.000	(0.000, 125.8)	0.581 ‖
10-HDHA [b]	20.5	(0.000, 197.11)	0.000	(0.000, 14.97)	<0.001‖
11-HDHA [b]	0.000	(0.000, 191.1)	0.000	(0.000, 0.000)	0.022 ‖
13-HDHA [b]	19.3	(0.000, 87.86)	0.000	(0.000, 0.000)	<0.001 ‖
14-HDHA [b]	41.3	(0.000, 299.69)	0.000	(0.000, 57.82)	<0.001 ‖
17-HDHA [b]	0.000	(0.000, 364.5)	0.000	(0.000, 0.000)	0.001 ‖
20-HDHA [b]	107.5	(44.09, 748.89)	25.4	(0.000, 105.46)	<0.001‖
19,20 DiHDPA [a]	1448	(1240, 1690)	1098	(878, 1374)	0.040

OxoODE, oxooctadecadienoic acid; HODE, hydroxyoctadecadienoic acid; EpOME, epoxyoctadecenoic acid; DiHOME, dihydroxyoctadecenoic acid; EKODE, epoxyketooctadecenoic acid; TX, thromboxane; HETE, hydroxyeicosatetraenoic acid; DHET, dihydroeicosatetraenoic acid; HETrE, hydroxyeicosatrienoic acid; HOTrE, hydroxyoctadecatrienoic acid; PG, prostaglandin; HEPE, hydroxyeicosapentaenoic acid; HDHA, hydroxydocosahexaenoic acid; MaR, maresin; DiHDPA,dihydroxydocosapentaenoic acid.

Results are expressed as mean (95% CI), except [a] geometric mean (95% CI), [b] median (lower and upper quartile) [†] Using independent samples *t*-test, except ‖ using Mann-Whitney U test.

4.7.6 Heart rate and heart rate variability

Twenty-four hour, day-time and sleep-time physical activity, heart rate and HRV are reported in **Table 15**. HRV parameters analysed from the data obtained from the Actiheart monitors are presented as IBI, time-domain, frequency-domain and non-linear parameters.

The average recording time for the 24 h analysis was 21:02 (95% CI 20:11, 21:52) hours, for the day time analysis was 13:08 (95% CI 12:38, 13:38) hours and for the sleep time analysis was 5:56 (95% CI 5:25, 6:27) hours. Statistical significance was obtained for the following parameters over 24 h analysis: vegans had higher mean SDNN (172 *vs.* 145 ms), SDANN (155 *vs.* 128 ms) and VLF (17980 *vs.* 12619 ms^2). Differences in these parameters indicate greater variability in longer-phase cycles in the vegan group during the 24 h period, whereas beat-to-beat (parasympathetically driven) variability (RMSSD, pNN50 and HF) and IBI/HR were not different between groups over the 24 h period. This is substantiated by much greater sleep-time minus day-time differences in mean IBI/HR, indicating that vegans experience a greater drop in HR from day to night compared to omnivores, due to having faster day-time HR.

In contrast with the 24 h measurements, HRV was reduced in vegans in the day-time (8 h segment) compared to omnivores, even after adjusting for physical activity and other covariates. Vegans had significantly lower IBI (721 *vs.* 787 ms), SDNN (101 *vs.* 121 ms), RMSSD (25 *vs.* 34 ms), pNN50 (2.7 *vs.* 6.6 ms), LF (628 *vs.* 908 ms^2) and HF (135 *vs.* 260 ms^2). Heart rate (reciprocal to IBI) was higher in vegans compared to omnivores but fell just short of statistical significance ($p = 0.062$).

From the sleep-time analysis (2 h segments), no statistically significant differences were found between groups for any of the HRV parameters. The sleep-time minus day-time differences in HR/IBI were statistically significant, with the mean decrease in HR/increase in IBI from day-time to sleep-time being distinctly larger in vegans compared to omnivores. Vegans also presented with larger increases in beat-to-beat HRV parameters (RMSSD, HF, pNN50) during nocturnal sleep and smaller decreases in overall (SDNN) and longer-phase HRV (VLF).

Table 15 – Physical activity, heart rate and heart rate variability parameters of study participants over 24 h, day-time and sleep-time, with sleep-time – day-time differences (n=47).

	Omnivore (n = 24)	**Vegan (n = 22*)**	***P*** [§]
Twenty-four hour measurements			
Activity, IBI and HR			
Physical activity (cpm)[a††]	236 (196, 284)	285 (233, 348)	0.181
IBI (ms)[††]	845 (798, 892)	811 (760, 861)	0.336
HR (bpm)[††]	75 (71, 79)	78 (74, 83)	0.210
Time-domain HRV parameters			
Ti	38 (33, 42)	41 (36, 46)	0.294
SDNN (ms)[††]	145 (129, 162)	172 (154, 189)	**0.039**
SDANN (ms)	128 (114, 143)	155 (139, 170)	**0.018**
RMSSD (ms) [a]	35 (31, 40)	35 (30, 40)	0.905
pNN50 (%)[a]	8.9 (6.5, 12.2)	7.0 (5.0, 9.7)	0.299
Frequency-domain HRV parameters			
LF (ms^2) [a]	971 (786, 1198)	761 (608, 953)	0.130
HF (ms^2) [a]	350 (262, 467)	309 (227, 421)	0.567
VLF (ms^2) [a]	12619 (10351, 15379)	17980 (14547, 22204)	**0.022**
Non-linear methods			
SD1:SD2 (Poincaré ratio)	0.13 (0.11, 0.14)	0.11 (0.10, 0.12)	0.051

	Omnivore (n = 24)	Vegan (n = 22*)	*P* [§]
Day-time (8 hours) measurement			
Activity, IBI and HR			
Physical activity (cpm) [a]	437 (347, 552)	443 (345, 570)	0.935
IBI (ms)	787 (745, 830)	721 (675, 766)	**0.039**
HR (bpm)	80 (76, 84)	86 (81, 91)	0.062
Time-domain HRV parameters			
Ti	31 (28, 34)	27 (24, 31)	0.135
SDNN (ms)	121 (109, 132)	101 (89, 113)	**0.021**
SDANN (ms)	100 (89, 111)	84 (73, 96)	0.056
RMSSD (ms)	34 (30, 38)	25 (20, 30)	**0.009**
pNN50 (%) [a]	6.6 (4.6, 9.4)	2.7 (1.8, 3.9)	**0.001**
Frequency-domain HRV parameters			
LF (ms^2)	908 (785, 1032)	628 (495, 761)	**0.004**
HF (ms^2) [a]	260 (198, 342)	135 (100, 181)	**0.002**
VLF (ms^2) [a]	8325 (6894, 10052)	6470 (5277, 7929)	0.078
Non-linear methods			
SD1:SD2 (Poincaré ratio)	0.15 (0.13, 0.16)	0.13 (0.11, 0.15)	0.125

	Omnivore (n = 24)	Vegan (n = 23)	*P* [§]
Sleep-time (2 hours) measurement			
IBI and HR			
IBI (ms)	965 (903, 1026)	991 (925, 1056)	0.568
HR (bpm)	64 (61, 68)	62 (59, 66)	0.475
Time-domain HRV parameters			
Ti	19 (16, 22)	18 (16, 21)	0.762
SDNN (ms)	78 (68, 88)	85 (75, 96)	0.324
SDANN (ms) [a]	42 (36, 51)	44 (37, 54)	0.717
RMSSD (ms) [a]	38 (32, 45)	44 (37, 53)	0.214
pNN50 (%) [a]	10.9 (7.0, 16.9)	11.7 (7.3, 18.7)	0.826
Frequency-domain HRV parameters			
LF (ms^2) [a]	882 (646, 1205)	902 (647, 1256)	0.925
HF (ms^2) [a]	403 (280, 580)	464 (315, 684)	0.601
VLF (ms^2) [a]	2881 (2215, 3744)	3519 (2661, 3744)	0.304
Non-linear methods			
SD1:SD2 (Poincaré ratio) [a]	0.27 (0.23, 0.32)	0.28 (0.24, 0.33)	0.740

	Omnivore (n = 24)	Vegan (n = 22*)	*P* [§]
Sleep-time – day-time differences			
IBI and HR			
IBI (ms)	183 (139, 227)	270 (223, 317)	**0.012**
HR (bpm)	-16 (-19, -13)	-24 (-28, -20)	**0.003**
Time-domain HRV parameters			
Ti	-12 (-15, -9)	-9 (-12, -6)	0.185
SDNN	-42 (-55, -29)	-20 (-33, -6)	**0.028**
SDANN	-52 (-65, -39)	-39 (-53, -25)	0.184
RMSSD (ms)	8 (1, 14)	22 (15, 29)	**0.006**
pNN50 (%)	6.4 (0.9, 11.8)	14.2 (8.5, 20.1)	0.058
Frequency-domain HRV parameters			
LF (ms^2) [b]	123 (-282, 461)	245 (5, 714)	0.235[‖]
HF (ms^2) [b]	127 (-12, 417)	234 (84, 988)	0.095[‖]
VLF (ms^2)	-5836 (-7418, -4255)	-3088 (-4746, -1431)	**0.025**
Non-linear methods			
SD1:SD2 (Poincaré ratio)	0.14 (0.10, 0.18)	0.16 (0.12, 0.20)	0.418

Cpm, counts per minute; IBI, interbeat interval; bpm, beats per minute; HRV, heart rate variability; Ti, total number of all NN intervals divided by the height of the histogram of all NN intervals; SDNN, standard deviation of all NN intervals; ms, milliseconds; SDANN, standard deviation of the averaged NN intervals, calculated from 5 min epochs; RMSSD, the square root of the mean of the sum of squares of differences between adjacent NN intervals; PNN50, percentage of adjacent NN intervals that differed by more than 50ms; LF, low frequency power; HF, high frequency power; VLF, very low frequency power; SD1:SD2, the ratio of the SD of beat-to-beat IBI variability (SD1) against the SD of long-term IBI variability (SD2).

Results expressed as estimated marginal means (95% CI), adjusted for gender, age, BMI and 24 h activity for 24 h HRV and sleep-time – day-time HRV, or 8 h activity for 8 h day-time, and adjusted for gender, age and BMI only for sleep-time. Sleep-time – day-time represents HR/IBI and beat-to-beat HRV during a standardized 2 h nocturnal sleep period minus a standardized 8 h day-time period, to indicate the difference between night and day.

[*]Missing data from 1 subject due to unusable day-time HRV recording.

[§] P value obtained using analysis of covariance for normally distributed raw or LN transformed data (adjusted for gender, age, BMI and activity for 24 h, day-time and sleep-time – day-time differences, and adjusted for gender, age and BMI only for sleep-time), except for sleep-time – day-time differences in HF, where [‖] denotes use of an unadjusted non-parametric test, the Mann-Whitney *U* test, where data remained not normally distributed following LN transformation. [a] denotes geometric means (95% CI) and [b] denotes medians (lower and upper quartiles).

[††] Only recordings >18 h included for 24 h physical activity, SDNN, IBI and HR data analysis, *n* 21 for omnivores and 19 for vegans.

4.8 Discussion

It was hypothesised that LC *n*-3 PUFA status would be associated with parameters of cardiac autonomic regulation and to investigate this we compared HRV parameters and LC *n*-3 PUFA status in vegans with an omnivore control group in a cross-sectional observational study. Vegans presented higher overall and longer-phase components of HRV over 24 h, but lower HRV, shorter IBI and higher HR in the day-time compared to omnivores. These results were substantiated by the fact that vegans had a greater sleep-time minus day-time differences in mean IBI/HR.

4.8.1 Subject characteristic

Vegan and omnivore subjects were successfully matched for age and BMI and there were no statistically significant differences in distribution of sex between groups, although the sex distribution wasn't fully balanced. The anthropometry measurements - waist circumference and % body fat - didn't differ between groups. Seated DBP didn't differ between groups but seated SBP was significantly lower in vegans compared to omnivores. Two large studies directly compared blood pressure between vegans and omnivores: both the Adventist Health Study-2 (AHS-2) and the EPIC-Oxford study reported lower SBP in vegans compared to omnivores (213,214). In contrast, vegans presented a higher resting HR as well as a significantly higher ambulatory daytime HR and, although not statistically significant, presented a higher ambulatory 24 h HR. Though there is no data published directly comparing vegan and omnivore subjects HR, there is evidence of increased concentrations of DHA in erythrocytes being associated with lower resting HR (215) and a meta-analysis of randomised, double-blind, placebo-controlled trials showed that consumption of fish oil reduced HR (216). The lower LC *n*-3 PUFA levels in vegan erythrocyte and plasma could be a possible explanation for their higher resting HR compared to omnivores.

4.8.2 Plasma and erythrocyte membrane fatty acid composition

Plasma and erythrocyte lipid fatty acid analysis were used as short- and long-term biomarkers of fatty acid intake, respectively, in order to assess adherence to a vegan diet. The vegan subjects have been on a vegan diet for at least 2 y which was also found to be the cut-off

considered for long-term vegans in other studies measuring the erythrocyte membrane fatty acids (217,218). There was a study in British vegans and vegetarians that didn't show significant differences in the proportion of plasma LC *n*-3 PUFA according to duration of the diet between vegans and vegetarians that followed the diet from 1 y to 20 y (159).

Plasma fatty acid analysis results showed a higher LA and ALA status in vegans compared to omnivores and erythrocyte fatty acid composition showed a higher LA status. Although the mean intake of ALA was greater in vegans compared to omnivores, the lack of a significant difference for erythrocyte ALA concentration may be explained by membrane saturation or by the greater LA intake of vegans resulting in a displacement of ALA (217). Vegan subjects showed significantly lower plasma and erythrocyte levels of DHA and EPA and a higher erythrocyte level of DPA *n*-3. These results were also shown by other published studies (159,218–220). The relatively lower intake of LA and the presence of preformed EPA and DHA in the diet of omnivores can explain the relatively higher proportion of EPA and DHA in the erythrocyte membrane and plasma compared with vegans. Because of the negligible intake of EPA and DHA, vegans are fully dependent on the endogenous synthesis of these fatty acids from ALA. The results show significantly higher levels of plasma ALA and LA in vegans compared to omnivores which suggest that the levels of EPA and DHA in plasma and erythrocytes found in vegans are derived from endogenous synthesis or, to some extent, from the triacylglycerols in adipose tissue, which has a half-life of 6 months to 2 years (221). There is evidence suggesting that humans can convert meaningful amounts of ALA to EPA and DHA when in the presence of a deficiency and this might explain the erythrocyte and plasma levels of EPA and DHA in vegans (157). Also, the fact that the duration of the diet didn't have an effect on the proportions of plasma EPA and DHA in a British vegan cohort, suggest that when excluding the foods with preformed EPA and DHA from the diet, the endogenous synthesis of EPA and DHA results in a low but stable plasma concentration of these fatty acids (159). The erythrocyte O3I in omnivores was double that in vegan (5.42 *vs*. 2.71 %). This index has been validated as a biomarker of tissue LC *n*-3 PUFA status (174). It has also been proposed as a marker for risk of death from coronary heart disease, with the greatest cardioprotection being

attributed to an O3I greater than 8% (205). The omnivore group in this study had an average O3I lower than 8%, and it was lower than the one reported in previous studies in UK populations (203,222,223). This could be attributable to a low overall fish-intake, as data from the latest National Diet and Nutrition Survey (NDNS) reported a mean oily fish consumption below the recommended amount in all age groups, with adults aged 19 to 64 consuming an average of 54 g per week (52 g for men and 54 g for women)[7]. An inverse relationship was observed between erythrocyte LA:ALA ratios and erythrocyte EPA content in vegans, supporting the existing evidence that higher dietary intakes of LA may inhibit the conversion of ALA to LC *n*-3 PUFA (224).

4.8.3 HRV measurements

It was hypothesised that vegans, who would have a lower LC *n*-3 PUFA status, would also display lower mean HRV. However, the observed differences between groups were more complex than hypothesised, mainly due to the divergence in night/day differences. Differences between groups in all primary outcome variables (HR/IBI, SDNN and RMSSD) were observed, but the inherent difference varied according to whether the analysis was performed over 24 h or only during day-time.

4.8.3.1 24 h measurements

Contrary to the hypothesis, vegans had higher overall (SDNN) and longer-phase components (SDANN and VLF) of HRV over 24 h, whereas IBI/HR and beat-to-beat variability (RMSSD, pNN50 and HF) were not different between groups over the 24 h period.

Circadian changes are a key determinant of variability in HR over 24 h, measured as SDNN. Differences in mean nocturnal sleep-time and day-time IBI are a significant factor in the size of the SDNN value. Hence the higher overall variability being substantiated by a greater sleep-time minus day-time differences in mean IBI/HR, indicating that vegans experience a greater drop in HR from day to night compared with omnivores, due to having faster day-time HR.

[7] National Diet and Nutrition Survey. Results from Years 1-4 (combined) of the Rolling Programme (2008/2009 – 2011/12)

The longer-phase components (SDANN and VLF) of 24 h HRV represent changes in HR in response to periodic fluctuations in neurohormonal and circadian physiology rather than beat-to-beat variability. The higher 24 h SDANN and VLF presented by vegans may represent either more pronounced neurohormonal rhythms in vegans, or reflect the higher discrepancy in HR and HRV between sleep- and day-time in vegans or could be due to higher overall activity levels. Although activity levels between vegan and omnivores were not significantly different, vegans presented a trend towards higher activity levels over the 24 h recording. Overall, it has been shown that vegans tend to exercise regularly (225) which is not necessarily reflected by the activity level registered over only one 24 h recording. Healthy physically trained subjects have been shown to have enhanced circadian variation which is reflected in increased parasympathetic activity of the longer-phase components of the HRV (226), and hence could have impacted the results.

4.8.3.2 Day- and sleep-time measurements

In line with the hypothesis, vegans had higher HR and lower IBI, overall (SDNN) and beat-to-beat HRV (RMSSD, pNN50 and HF) during day-time. The lack of difference in sleep-time HR between groups, together with observations of shorter IBI (and non-statistically significant greater HR) during the day in vegans, suggests that the larger night-day difference in vegans is a result of greater circadian fluctuations in sympathetic–parasympathetic balance. Sleep-time minus day-time HRV also reflects the degree of circadian modulation of autonomic regulation of HR in both vegans and omnivores. The larger increases in beat-to-beat HRV parameters (RMSSD, HF, pNN50) during nocturnal sleep may indicate a greater suppression of parasympathetic regulation during day-time waking hours in vegans when considered alongside the shorter mean day-time IBI in this group compared with omnivores. These observations might indicate that low LC *n*-3 PUFA status could lead to either a predominance of sympathetic regulation, a greater withdrawal of parasympathetic activity, or eventually, due to depletion of LC *n*-3 PUFA in the cardiomyocyte membranes, there could be a greater stimulation of pacemaker activity despite normal levels of sympathetic neural activity during waking hours (227). Nevertheless, there is the possibility that differences in HRV observed between vegans

and omnivores are unrelated to LC *n*-3 PUFA tissue status, although to test this possibility a dietary intervention trial would be required. In animal models the evidence suggests an effect of DHA in reducing HR via modulation of pacemaker activity rather than changes in cardiac autonomic regulation (84).

To date, there has only been one published study assessing the effects of long-term vegetarian diets (> 2 y) on cardiac autonomic function, using frequency-domain HRV analysis, in healthy postmenopausal Chinese women (228). This study reported a higher day-time resting HF and LF power in vegetarians compared to non-vegetarians which is contrary to the results of this study. However, the HRV recording length of the postmenopausal Chinese women was 5 minutes against 8 h in our day-time analysis.

4.8.4 Lipid mediators and inflammatory markers

The novel data derived from the lipidomic analysis allowed observation of different patterns of fasting plasma concentrations of lipid mediators in vegans and omnivores. Vegans, representing a population with no dietary intake of LC *n*-3 PUFA, presented lower or zero concentrations of DHA- and EPA-derived SPM precursor markers of resolvins (18-HEPE, 17-HDHA), and maresins (14-HDHA), lower concentrations of markers of AA-derived prostanoid production (6-keto $PGF_{1\alpha}$ – a marker of PGI_2 synthesis, and 13,14-dihydro $PGF_{2\alpha}$/13,14-dihydro-15-keto $PGF_{2\alpha}$ – markers of $PGF_{2\alpha}$ production) and greater concentrations of ALA- and LA-derived lipid mediators compared to omnivores. There were no differences between groups for a range of AA-derived LOX-catalysed mediators (HETE), suggesting that the lipid mediator profile of vegans may not necessarily be entirely pro-inflammatory relative to omnivores (227). 17-HDHA (DHA-derived precursor marker of neuroprotection D1 – NPD1 – and RvD1) was not detected in the fasting plasma of any vegan subjects, whereas nine out of twenty-four omnivores had detectable concentrations. There were also marked differences in concentrations of 18-HEPE (EPA-derived precursor marker of RvE1) and 14-HDHA (DHA-derived precursor marker of MaR1). These same precursor markers concentrations were found to be increased following LC *n*-3 PUFA supplementation in healthy adults (229). The role of NPD1 in protecting the nervous system from inflammation-related injury shows that DHA-dependent

physiological mechanisms exist in synapses and neural circuits in order to sustain neuronal function (230,231). The higher levels of LA-derived lipid mediators in vegans is likely due to higher dietary intakes, which is supported by the FFQ estimates of dietary intake, proportions of total plasma fatty acids and incorporation into erythrocyte membrane lipids (232,233). Although plasma ALA proportions were higher in vegans, reported dietary intakes didn't differ. However, FFQ estimates of dietary intakes are likely to underestimate true intakes due to incomplete food composition data. LA-derived lipid mediators, namely 9- and 13-HODE, and 9- and 13-oxoODE have been implicated in a variety of diseases, including CVD, and lowering dietary LA was shown to reduce the levels of these bioactive LA-derived metabolites (232). On the other hand, ALA-derived lipid mediators, including 13-HOTrE, have been shown to have cytoprotective effects in cell experiments (234).

Although no differences were found in IL-6 concentrations, an inflammatory marker, between vegans and omnivores, this does not necessarily indicate that there are no differences between groups in their capacity to inhibit or resolve acute inflammatory events, since circulating cytokine concentrations have limited utility as biomarkers of inflammation that may be occurring in localised areas of tissue (227). Previous studies have shown that serum IL-6 concentrations were inversely correlated with HRV in men with renal disease (93), men with the metabolic syndrome (235) and young healthy subjects (88), although not all studies agree (89). Down-regulation of inflammatory cytokine gene expression plus increased production of pro-resolving lipid mediators are two potential mechanisms whereby cardiac function might possibly be modulated by increased EPA and DHA intakes, by reducing inflammatory tissue damage in the brain and autonomic nerves, and also in the heart tissue itself.

4.8.5 Biochemistry profile and dietary intake

The biochemistry profile of vegans presented significantly lower levels of total cholesterol and LDL cholesterol with no significant differences in the levels of glucose, triacylglycerol, HDL cholesterol or haemoglobin. A lower total and LDL-cholesterol levels have been previously reported in studies comparing vegans with omnivores (236–238). This could be associated with reported dietary intakes of SFA (%E) in vegans being almost half the

dietary intake of omnivores, in agreement with results reported in larger vegan populations (239), and correspondingly lower amounts of palmitic acid as a proportion of total plasma fatty acids. However, these differences are less likely to have a major influence on cardiac electrophysiology since animal studies have shown that PUFA feeding decreased vulnerability to arrhythmia compared with high SFA feeding without any reduction in the proportion of membrane SFA, and high-MUFA feeding did not reduce arrhythmia compared with high-SFA diets (240). This suggests that SFA membrane composition is not a major determinant of vulnerability to arrhythmias and addition of LC n-3 PUFA (replacing mainly oleic acid and n-6 PUFA) might be the most important determinant. In fact, erythrocyte SFA proportions were not significantly different between groups and vegans presented lower daytime HRV, and therefore potentially a greater risk of arrhythmia if there was also coronary atherosclerosis present, despite lower SFA intake (227). There was one study in post-menopausal women that showed a significant moderate negative correlation between LDL and HRV (LF and HF power) measured over 5-min rest (53). However, the strength of correlations found between LDL and HRV parameters in the present study were weak (data not shown).

The dietary intake of vegan subjects varied significantly from the omnivore subjects, with significantly lower intakes of protein, saturated fat, EPA and DHA and significantly higher intakes of carbohydrates, PUFA and LA, in accordance with other studies results (236,241,242). Although intake of vitamin B_{12} was significantly lower in vegan compared to the omnivore subjects, serum concentrations were not statistically significantly lower. This might be because supplements were not included in the FFQ analysis due to incomplete information provided about the type of some supplements. In this study, all subjects had serum concentrations of vitamin B_{12} within the normal ranges (180-1100 ng/L), as defined by the clinical biochemistry department at KCH, except for an omnivore participant that presented a concentration of 1168 that could be explained by the intake of vitamin B_{12} rich supplements. Broad cut-off points for vitamin B_{12} deficiency have been reported by different authors which makes comparison of prevalence of vitamin B_{12} deficiency with other published studies difficult (243). Although the lower serum concentrations of vitamin B_{12} in vegans not obtaining a statistically significant

difference from omnivores, the results agree with previous studies showing a lower average serum concentration of vitamin B_{12} in vegans compared to omnivores (244). Vitamin D levels were not significantly different between groups, which is likely to be due to the significantly higher vitamin D supplement use in vegans compared to omnivores. Nevertheless, the nature of the study design doesn't allow to exclude the influence of other dietary or lifestyle factors on HRV related with the vegan lifestyle.

4.8.6 Strengths and limitations of the present study

The strengths of this study include: 1) the distinctive type of population studied, since vegan diets are naturally free from EPA and DHA, representing a model of LC *n*-3 PUFA tissue deficiency; 2) measurement of plasma and erythrocyte membrane LC *n*-3 PUFA, which complements the data obtained from the FFQ as a biomarker of dietary intake; and 3) the novelty of measuring the lipid mediator profile in unsupplemented dietary *n*-3 PUFA free-living humans.

The limitations of this study include the cross-sectional design, which limits the findings to being exploratory in nature and the associations between low LC *n*-3 PUFA status and reduced HRV requiring confirmation by a RCT of EPA + DHA supplementation in a population with a low O3I (<3%). On the other hand, the FFQ has only been previously validated in the general population and therefore not specific for the vegan diet, which might have over or underestimated certain nutrients intake. The FFQ presents limitations related to recall bias and measurement errors, including the errors derived from assuming a set portion size for each food group amongst others. However, from a subset of participants (12 omnivores and 8 vegans) that completed 4-day food diaries, the nutritional analysis obtained from these (data not shown) supported the data obtained with the FFQ. Although statistical power calculations were carried out for the primary HRV outcomes, the study may be underpowered to detect group differences in other variable outcomes such as beat-to-beat HRV. Multiple statistical testing was performed to explore group differences in short- and long-term, as well as time- and frequency-domain HRV, increasing the risk of generating false-positive results. There is no agreed upon method for correcting statistical analyses that involve the full set of HRV measures, but the data set

represents groupings of related outcomes rather than a large collection of disparate variables. The data presented here are consistent when comparing variables that represent similar physiological phenomenon. For example, there are two time-domain (RMSSD, pNN50) and one frequency-domain (HF) parameters of beat-to-beat variability. These are all vagally regulated and all show consistently that day-time beat-to-beat HRV is lower in vegans compared with omnivores. Therefore, although type I errors cannot be ruled out with complete certainty, it is reassuring that statistically significant differences between groups are supported by analogous parameters. Although there were no statistically significant differences in sex distribution between groups, matching for sex was not fully achieved. Nevertheless, any influence of this imbalance in sex distribution on HRV results was minimised by adjusting for sex in addition to age, BMI and activity levels for 24 h and day-time HRV. Technical problems in obtaining good quality sleep-time HRV data limited the standardised duration of sleep-time HRV to 2 h which may have led to effects on longer phase-HRV parameters being missed.

In conclusion, this study shows differences in the cardiac autonomic regulation in vegans and omnivores with vegans presenting higher HRV over 24 h due to greater sleep-day differences compared to omnivores. This was mostly due to vegans presenting relatively lower HRV in the day-time rather than higher HRV at night, as no sleep-time differences were observed. This could indicate a greater predominance of sympathetic activity with reduced parasympathetic activity during waking hours due to an exaggerated response to various daily events and/or activities, or could be a direct effect of a low proportion of LC *n*-3 PUFA in the membrane of cardiomyocytes that result in altered cardiac function when exposed to stimulation conditions.

Chapter 5 Heart rate variability and long chain *n*-3 polyunsaturated fatty acids in chronic kidney disease patients on haemodialysis: a cross-sectional study

5.1 Introduction

Cardiovascular disease is particularly prevalent in CKD (44), and accounts for 43% of all-cause mortality among dialysis patients (45), compared to an estimated 11% of deaths in the general population (245). It has been observed that the risk of SCD is doubled when a patient with CKD stage 5 starts dialysis (46) and in haemodialysis, SCD accounts for two thirds of all cardiac deaths and one fourth of all-cause mortality (45).

Haemodialysis patients have lower serum LC *n*-3 PUFA levels compared to control populations without CKD (246,247), and lower serum LC *n*-3 PUFA : AA ratios were predictive of CVD events (247). Fish consumption has been associated with reduced mortality in an observational study with incident dialysis patients (248), and Friedman et al suggested an inverse association between erythrocyte LC *n*-3 PUFA levels and mortality in haemodialysis patients (249). Although evidence from RCT in end stage renal disease is essential before recommendations are made, preliminary observational data indicates that supplementation with LC *n*-3 PUFA (20:5*n*-3, EPA and 22:6*n*-3, DHA), may have the potential to offer a safe, side-effect-free therapy to reduce SCD risk in patients initiating haemodialysis. Clinical trials that addressed SCD as a primary outcome in non-CKD populations are scarce due to low statistical power in most study populations. The "OMEGA" multicentre RCT defined SCD as a primary outcome but reported no reduction in risk in myocardial infarction survivors following LC *n*-3 PUFA supplementation on top of modern guideline-adjusted medication, with low SCD rates of 1.5% in both intervention and placebo groups, and post-hoc calculated statistical power at only 44% (150,250). The earlier, pre-statin era GISSI-Prevenzione trial, in 11,323 myocardial infarction survivors, showed a significant reduction in SCD after just 4 months of treatment (0.88 g/d EPA+DHA) (251). The adequately-powered GISSI-Heart Failure trial demonstrated a non-significant relative risk of 0.93 in 6975 chronic congestive heart failure patients (average 3.9 y follow-up, 0.88 g/d EPA+DHA) (141).

End-stage CKD patients are a population with low HRV indicating autonomic dysfunction (252) and low HRV has been reported to be independently associated with increased risk of all-cause mortality, SCD and all cardiac death in haemodialysis patients (253–255). The pathogenesis of CVD in patients with CKD differs from the general population due to the prevalence of non-traditional risk factors, such as oxidative stress, inflammation, endothelium dysfunction and altered electrolyte balance (256) which results in lower effectiveness of the current treatments targeting traditional cardiovascular risk factors (such as statins, blood-pressure lowering and anti-platelet drug therapy) (257) and the need to explore other treatment options. LC *n*-3 PUFA presents as a good candidate for risk prevention in this population due to their potential anti-inflammatory, anti-oxidative and anti-arrhythmic effects on the cardiac myocytes (84,258,259). Mixed results have been obtained in previous investigations into the effect of LC *n*-3 PUFA on HRV in healthy subjects but there is stronger evidence in myocardial infarction survivors of benefits from fish oil supplementation (260). Few studies have investigated the relationship between LC *n*-3 PUFA and HRV in CKD patients. A pilot RCT in patients undergoing dialysis (n = 29) tried to assess the effects of fish oil supplementation on HRV but due to the high number of drop-outs (4 and 6 patients in the intervention and control groups, respectively), the two groups were not compared. Instead, the remaining patients with good quality Holter recordings (n = 17) were used to assess the relationship between SDNN and LC *n*-3 PUFA content in granulocytes. The findings showed a positive correlation between the content of EPA, DHA and total LC *n*-3 PUFA, and SDNN in these patients (106). A subgroup (*n* = 30) of the chronic haemodialysis patient population used in a parallel design study on *n*-3 PUFA and cardiovascular events (261), was used to determine the effect of 1.7 g/d EPA+DHA on HRV after 3 months of intake (111). HRV parameters were found to be unaffected after 3 months supplementation, although this study was hampered by lack of statistical power, short duration, and the limited range of HRV parameters measured. Due to the equivocal findings and lack of studies investigating the relationship between LC *n*-3 PUFA and HRV as a primary outcome, at present it is unclear whether low EPA+DHA tissue status (as indicated by the erythrocyte O3I) in end stage renal disease patients is associated with

HRV, and whether supplementation to increase EPA+DHA tissue status would be effective in improving autonomic function and cardiovascular outcomes.

5.2 Hypothesis

It is hypothesised that the proportion of EPA+DHA in the erythrocyte membrane – a biomarker of longer-term dietary intake – will be independently positively associated with HRV in patients with CKD stage 5 who have recently commenced haemodialysis.

5.3 Aims

This study aimed to test the feasibility of applying this cross-sectional study design on a larger scale by 1) establishing the viability of measuring erythrocyte membrane LC *n*-3 PUFA content as a biomarker of longer-term dietary intake, and 2) characterising the variability of LC *n*-3 PUFA status in patients who have recently commenced haemodialysis, since low variability in the population would preclude meaningful statistical tests of associations with HRV. The study also aimed to collect pilot data on relationships between erythrocyte membrane LC *n*-3 PUFA status and HRV in these patients.

5.4 Methods

5.4.1 Study design

This was a cross-sectional pilot study that characterised the tissue LC *n*-3 PUFA status of CKD patients who have recently started haemodialysis treatment and explored a potential relationship with HRV parameters.

5.4.2 Ethics and consent

Ethical approval was obtained from NRES Committee London – Camberwell St Giles (REC ref: 14/LO/0186). The study was registered on clinicaltrials.gov (NCT02014792) and conducted at King's College Hospital (KCH) and Guy's and St Thomas' Trust (GSTT) and their respective satellite units. **Figure 19** shows the locations visited for recruitment, taking consent from the patients and running the study days. The study was conducted between July 2014 and January 2016.

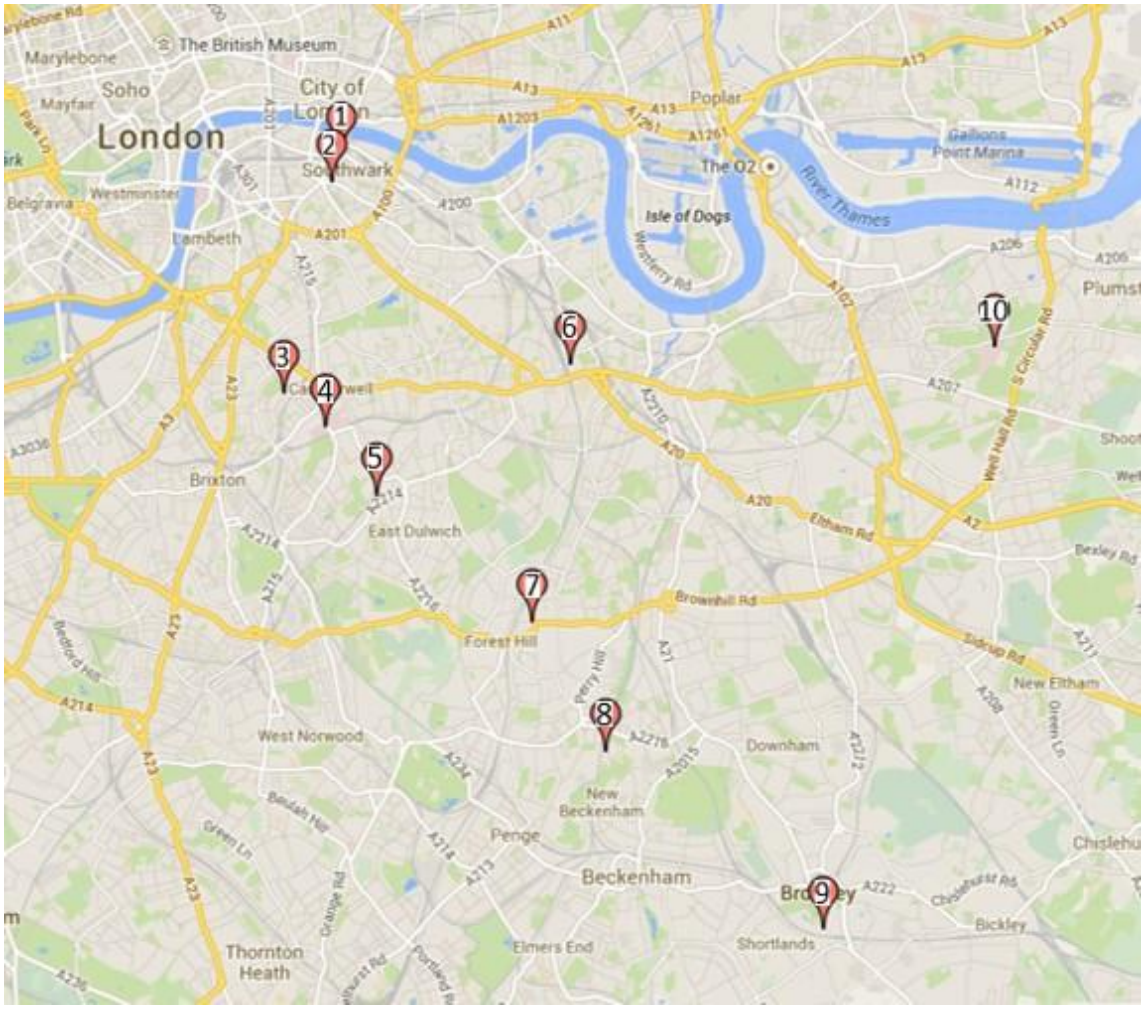


Figure 19 – Location of the units included in the study. Legend:
1 – Astley Cooper Dialysis unit, Guy's hospital 2 – Borough Kidney Treatment Centre
3 – Camberwell satellite dialysis unit 4 – Victor Parsons Haemodialysis Unit, KCH
5 – Dulwich satellite dialysis unit & Dulwich mobile dialysis unit, Dulwich community hospital
6 - New Cross Gate Satellite Dialysis Unit 7 – Forest Hill Satellite Dialysis Unit
8 – Sydenham Satellite Dialysis Unit 9 – King's at Bromley Satellite Dialysis Unit
10 – Woolwich Satellite Dialysis Unit, Queen Elizabeth Hospital

5.4.3 Outcome measures

The primary outcome of the study was the relationship between HR and HRV parameters during haemodialysis - more specifically 24 h SDNN and triangular index, which are reflective of overall HRV - and the proportion of EPA + DHA (O3I) in the erythrocyte membrane. The secondary outcomes include other time- and frequency-domain, and non-linear HRV parameters, during 2 h dialysis, 24 h and sleep-time, plasma proportions of EPA, DPA and DHA, background diet (using food frequency questionnaires) and clinical risk factors, including history of sleep apnoea or suspected sleep apnoea (using Epworth Sleepiness Scale and Berlin Questionnaire, described in section 5.5.3), waist circumference, 12 month average SBP and

DBP and BP variability prior to starting haemodialysis, hs-CRP at start of dialysis session, serum creatinine, GFR, albumin, sodium, potassium, phosphate and calcium before and after the dialysis session; to assess the contribution of these risk factors to any relationships observed between HRV and erythrocyte membrane LC *n*-3 fatty acid composition.

5.4.4 Inclusion and exclusion criteria

The inclusion criteria were women and men with CKD stage 5 commencing haemodialysis (6 to 10 weeks after the first session), aged 40-80 years and able to provide written informed consent.

The exclusion criteria included unwilling to participate, history of chronic liver disease or neuropathy, infection or antibiotics within the last month and history of prior renal replacement therapy including transplantation.

5.4.5 Participants and recruitment

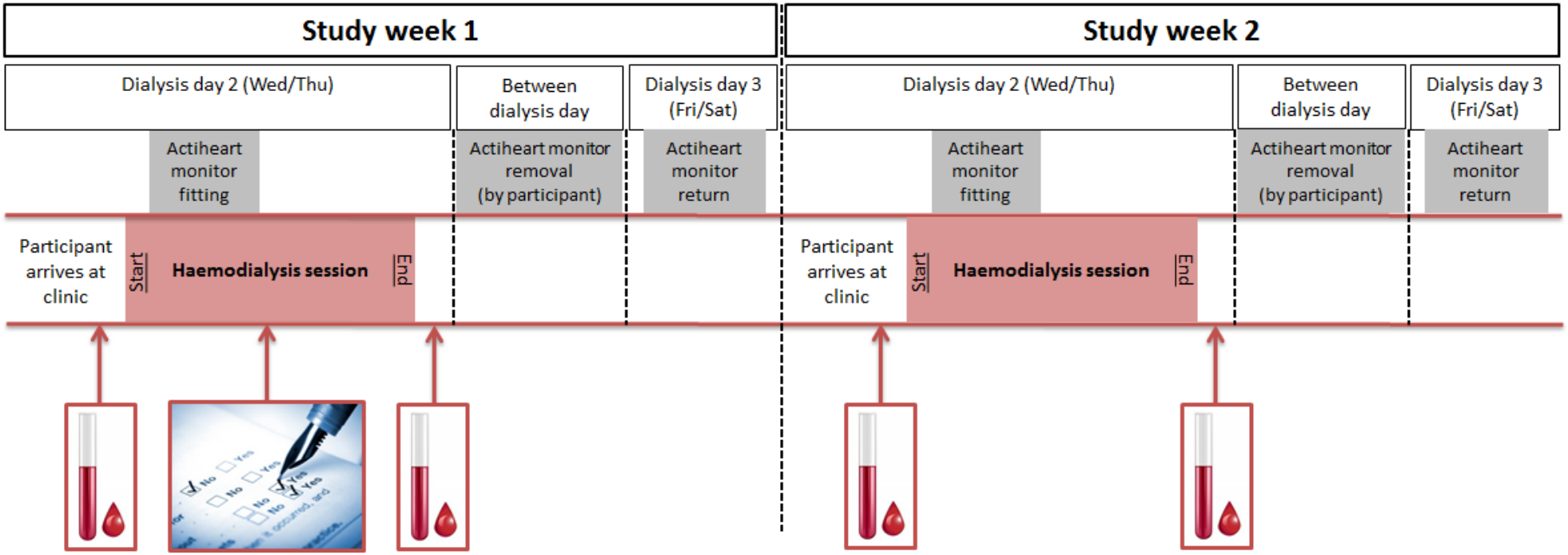
Potential participants were patients under the care of a Nephrologist. Only members of the clinical care team did the electronic search of patient records for suitable patients for the study and to check whether they met the inclusion criteria. The study team included members of the renal clinical team, who were associated with the study directly. Patients were identified through the course of usual clinical care, or via an efficient electronic database search conducted by Dr Helen MacLaughlin and Dr. Nicola Kumar (and Dr. Jasmine Lee while Dr. Kumar was on maternity leave) at King's College Hospital and Guy's and St Thomas' Trust, respectively. Once a list of potential eligible patients was obtained, the nurses would approach the patient first asking if they were willing to talk to the PhD student, and then the student would approach each patient to give an explanation about the study and leave them with a patient information sheet (**Appendix 6**) before coming back another day to answer any questions the patient may have about the study and obtain written informed consent. Once consent was obtained, the study visits were scheduled for all the outcome measurements. Patients had haemodialysis treatment three times a week for a varied prescribed amount of time (usually 3 h to 4 h) and these occurred in two different schedules, Monday, Wednesday and Fridays or Tuesday, Thursdays and Saturdays where the treatment could happen in the morning, afternoon or evening. The patients

would have to enrol in the study shortly after commencing haemodialysis treatments (and the study days were chosen in order to avoid the session right after the two consecutive days of intradialytic period, also known as the "long gap" (Monday or Tuesday), when uraemia was increased, and to maintain consistency throughout the study). Once the consent was obtained, the two study dates were agreed with the patients and then added to the patient's medical diary so that the nurse in charge would be aware of when blood samples (pre- and post-dialysis) were needed.

5.5 Study days

An overall outline of the study days can be found in **Figure 20**. In both study visits, pre- and post-dialysis blood sample were taken by a nurse to measure electrolytes, including sodium, potassium, phosphate and calcium; and hs-CRP and to measure erythrocyte membrane and plasma fatty acid composition in pre-dialysis blood only. Waist circumference was taken on patient's arrival on the first study visit before going on the dialysis machine. Immediately after the nurse started the patient on haemodialysis treatment, the PhD student would fit the monitor to record HR and HRV for a 24 h period. Patients were also asked to complete a FFQ and an Epworth sleepiness scale and Berlin questionnaire in the first study visit. Additional information was obtained from the medical records including patient's medical history, ethnicity, date of birth, pre- and post-dialysis weight and fluid removal in study days, current medication, any side effects or serious adverse events during the first 6 weeks of haemodialysis, and the previous 12 month average SBP and DBP (before starting haemodialysis). All patients continued with the best medical care, as appropriate, at the nephrologist's discretion.

Figure 20 - Overall study days outline.



5.5.1 HRV measurements

Heart rate variability was measured using the Actiheart monitoring equipment (CamNtech Ltd, Cambridge, UK). Two ECG electrodes (SP-50, 50 mm round, Pulse Medical) were placed on the chest after skin preparation to fit the Actiheart monitor. A short signal test of around 5 minutes was performed before programming for the 24 h recording to confirm that the level of R wave signal being picked up by the Actiheart was adequate. Data processing of the 24 h HRV recordings was carried out using the Actiheart software (version 4.0.91, CamNtech Ltd, Cambridge, UK) and Kubios HRV analysis software (Biosignal Analysis and Medical Imaging Group, Department of Physics, University of Kuopio, Finland). For more detailed information please refer to the general methods chapter (**Chapter 2, section 2.1**). Patients were given an activity and event diary (**Appendix 7**) to complete with information about the duration of dialysis, type of activities done, and nap and sleep times at night. HRV and HR/IBI data were analysed for a standardised 2 h of haemodialysis (from the moment the monitors were fitted), for the full length of recording time (minimum of 18 h, up to 24 h) and sleep time.

5.5.2 Dietary assessment

The EPIC-Norfolk FFQ (pages 2 to 10) was used in this study to assess patients' background diet over the past 12 months. Further details about the questionnaire can be found in **Chapter 4 (section 4.5.3)**.

5.5.3 Epworth sleepiness scale and Berlin questionnaire

The Epworth Sleepiness Scale (ESS, **Appendix 8**) is a validated method of assessing the likelihood of falling asleep in a variety of situations. The maximum score is 24 and can be used to clinically subdivide the patients into either the normal range (ESS<11), mild subjective daytime sleepiness (ESS=11-14), moderate subjective daytime sleepiness (ESS=15-18) or severe subjective daytime sleepiness (ESS>18) (262).

The Berlin Questionnaire (**Appendix 8**) asks about risk factors for sleep apnoea, namely snoring behaviour, wake time sleepiness or fatigue, and the presence of obesity or hypertension. Predetermination of high risk and lower risk for sleep apnoea was based on responses in three symptom categories. In category 1, high risk was defined as persistent symptoms (>3 to 4 times

/ week) in two or more questions about their snoring. In category 2, high risk was defined as persistent (>3 to 4 times / week) wake time sleepiness, drowsy driving, or both. In category 3, high risk was defined as a history of high blood pressure or a BMI more than 30 kg/m^2. To be considered at high risk for sleep apnoea, a patient had to qualify for at least two symptom categories.

5.5.4 Blood sample collection, handling and analysis

Pre- and post-dialysis blood samples collected in SST^{TM} tubes were used to determine serum sodium, potassium, phosphate and calcium as well as albumin, creatinine and eGFR (estimated GFR according to CKD-EPI formula) before and after dialysis session and sCRP before dialysis. These analyses were performed by a clinical pathology accredited clinical biochemistry laboratory (ViaPath, Kings College Hospital). Pre-dialysis blood samples collected in EDTA tubes were used to determine erythrocyte membrane fatty acid composition (in percentage weight) and plasma fatty acid composition (concentration and percentage weight). These analyses took place at King's College London and were performed by the student with the help of technician Robert Gray.

5.6 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 21.0 (Statistical Product and Service Solutions; IBM Corp.). Paired *t*-tests and Pearson correlations between first and second visits were performed for HR/IBI, HRV measurements and erythrocyte membrane and plasma fatty acid composition to test for intra-subject reproducibility. Subsequently the values were averaged in all subjects that had recordings for both visits for the final analysis. Pearson correlation coefficients were used to test the strength of correlations between variables in haemodialysis patients. Non-normally distributed data were normalised by natural logarithm (LN) before performing the correlations. Chi-square (χ^2) test and independent t-tests were used to compare categorical and continuous variables, respectively, between haemodialysis patients and a healthy cohort. Non-normally distributed data were normalised by LN transformation (results shown as geometric means and 95% CI) before analysis by independent t-test. If LN transfor-

mation failed to yield a normal distribution, a Mann–Whitney *U* test was applied to compare groups (results shown as medians with lower and upper quartiles).

5.7 Results

Figure 21 shows the flow of participants through the study. Of the 49 patients consented, there were 3 withdrawals due to 2 patients not feeling well before the start of the first study day and 1 patient that was transferred out to a unit that was not associated with KCH or GSTT. One patient became ineligible due to start of peritoneal dialysis before the first study visit. Forty-five patients completed the first study visit and 31 completed both study visits. The reasons for patients not completing the second study visit were: unwilling to carry on the study, study visits rescheduling problems, patient transfers, and patients becoming unwell. Of the 45 patients, 39 patients had good quality HRV recordings to be included in the 2 h dialysis period, 40 had good quality sleep-time HRV recordings (minimum 3 h period) and 35 had good quality 24 h recordings (minimum 18 h period). There was only one patient where it was not possible to obtain erythrocyte membrane fatty acid composition due to technical issues.

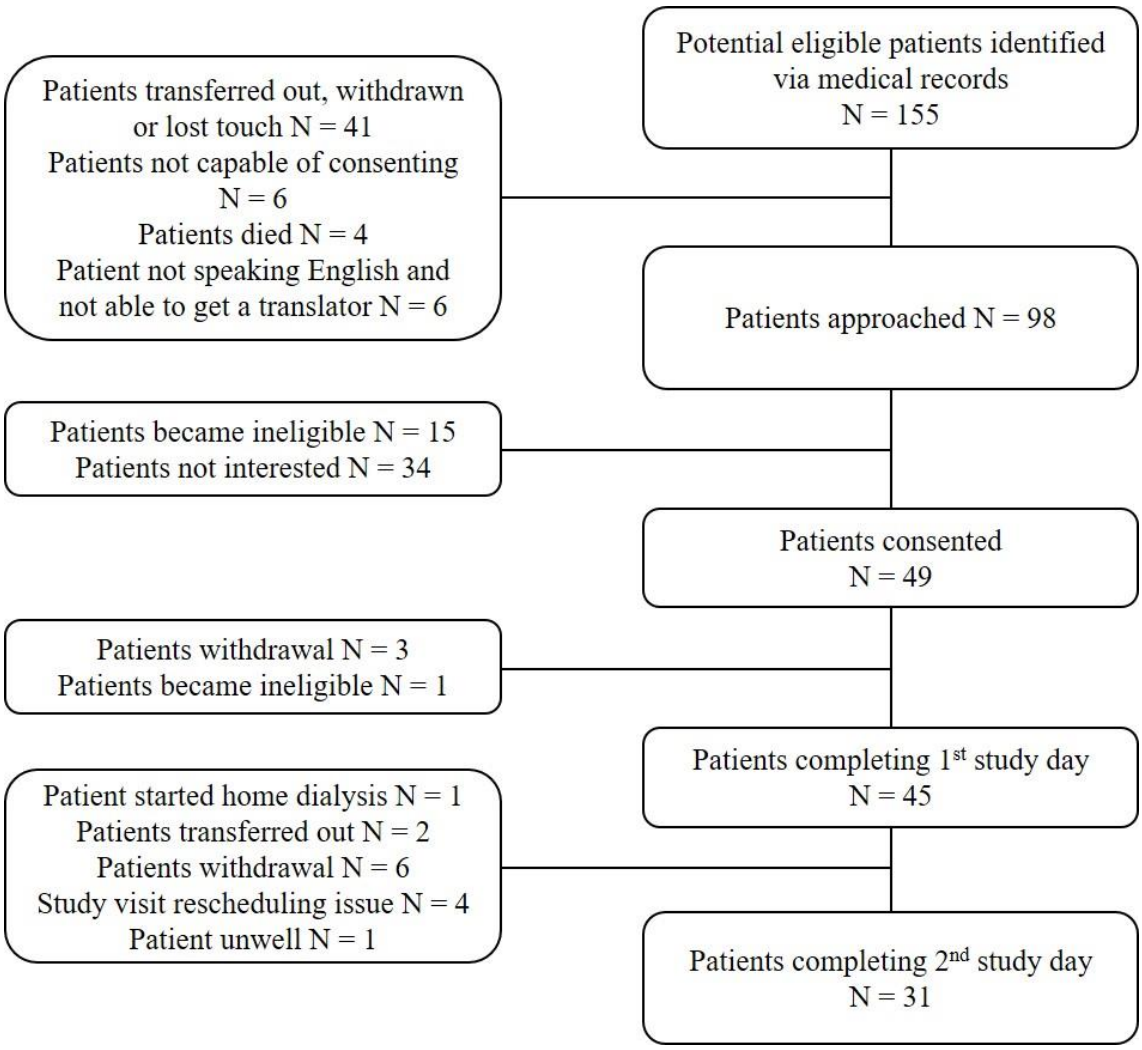


Figure 21 - Consort diagram.

Characteristics of the patients enrolled in the study are presented in **table 16**. Data were not equally available for all patients either due to unwillingness to complete the questionnaires/ undergo certain measurements, because the data were not available electronically on the medical records, or due to issues taking a blood sample or transporting samples by medical courier. For this reason, the number of data points available for each variable are displayed on the table. Most patients presented hypertension and the second most common co-morbidity was type 2 diabetes. Nearly half the patients were taking statins, β-blockers and iron injections and more than half were taking vitamin D supplements.

Table 16 - Characterisation of the study population (n=45).

Study population (n=45)				
	Mean	**SD**	**N**	**%**
Age (years)	58	9		
Sex				
Male			25	55.6
Female			20	44.4
Ethnicity				
White			20	44.4
Black			19	42.2
Other			6	13.3
Waist Circumference (cm)				
Male	105.6	13.2	17	
Female	97.1	19.4	12	
BMI (kg/m^2)	27.8	7.0	45	
Average SBP (mmHg)*	141	16	33	
Average DBP (mmHg)*	76	9	33	
Fluid removal (L)	2.0	0.8	45	
Pre- and post-dialysis measurements [1]			42	
Pre-dialysis creatinine (μnol/L)	585	192		
Post-dialysis creatinine (μnol/L)	228	100		
Pre-dialysis albumin (g/L) [b]	38 (36, 40)			
Post-dialysis albumin (g/L) [b]	40 (37, 44)			
Pre-dialysis sodium (mmol/L) [b]	139.3 (137.6, 141.0)			
Post-dialysis sodium (mmol/L) [b]	138.0 (137.0, 139.0)			
Pre-dialysis calcium (mmol/L)	2.17	0.15		
Post-dialysis calcium (mmol/L)	2.25	0.19		
Pre-dialysis potassium (mmol/L) [a]	5.1 (4.9, 5.4)			
Post-dialysis potassium (mmol/L) [a]	3.8 (3.6, 3.9)			
Co-morbidities [2]				
Hypertension			40	90.8
Coronary artery disease			5	11.4
Peripheral vascular disease			2	4.5
Heart failure			3	6.8
Cerebrovascular disease			3	6.8
Type 2 Diabetes			17	38.6
Hyperlipidaemia			9	20.5
Anaemia			5	11.4

	Mean	SD	N	%
Medications				
ACE inhibitor			11	25.0
ARB			10	22.7
β-blocker			20	45.5
α-blocker			12	27.3
Diuretic			10	22.7
Statin			21	47.7
Insulin			11	25.0
Anti-diabetic			6	13.6
Anti-depressant			6	13.6
Vitamin D			25	56.8
Calcium-based phosphate binders			18	40.9
Iron injections			21	47.7
Subjective daytime sleepiness			31	
Normal			23	74.2
Mild			5	16.1
Moderate			1	3.2
Severe			2	6.5
Risk of sleep apnoea			31	
High			20	64.5
Low			11	35.5
Fish and seafood intake (times/week)			35	
All fish and seafood	2.4	2.3		
Oily fish	0.6	0.6		
Nutritional intake			34	
Energy (kcal)	1846	889		
Protein (%E)	17.3	4.3		
Carbohydrate (%E)	47.4	8.3		
Total fat (%E)	34.4	6.8		
SFA (%E)	12.7	3.1		
MUFA (%E)	13.2	3.0		
PUFA (%E)	5.9	1.7		
20:5n-3, EPA (g)	0.14	0.11		
22:6n-3, DHA (g)	0.44	0.34		

* 12 months prior to commencing HD [a]Geometric means (95%CI) [b] Median (IQR)
ACE, angiotensin converting enzyme; ARB, angiotensin II receptor antagonists; BMI, body mass index; DBP, diastolic blood pressure; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; %E, percentage of energy; PUFA, polyunsaturated fatty acids; SBP, systolic blood pressure; SFA, saturated fatty acids. [1] eGFR measures were not included in the table as these are not valid in haemodialysis patients. [2] Information taken from medical records with no further details on the approach used to diagnose.

As expected, there were no significant differences between visit 1 and visit 2 for all HRV parameters during dialysis (n = 23), sleep time (n = 24) and 24h (n = 19), and also for erythrocyte membrane EPA, DHA and O3I (n = 26) and plasma EPA and DHA (n=31). All HRV parameters were significantly correlated with the same HRV parameters between the two study visits for dialysis (range of strength of correlations was moderate to strong: $0.578 < r < 0.880$

and *p* values < 0.01), sleep-time (range of strength of correlations was moderate to strong: 0.468 < r < 0.913 and *p* values < 0.05) and 24 h (range of strength of correlations was moderate to strong: 0.630 < r < 0.812 and *p* values < 0.01). Significant moderate to strong correlations were found between the two study visits measurements of erythrocyte membrane EPA (r = 0.687, *p* < 0.001), DHA (r = 0.497, *p* = 0.010) and O3I (r = 0.538, *p* = 0.005) and plasma EPA (r = 0.729, *p* < 0.001), DPA(*n*-3) (r = 0.907, *p* < 0.001) and DHA (r = 0.885, *p* <0.001).

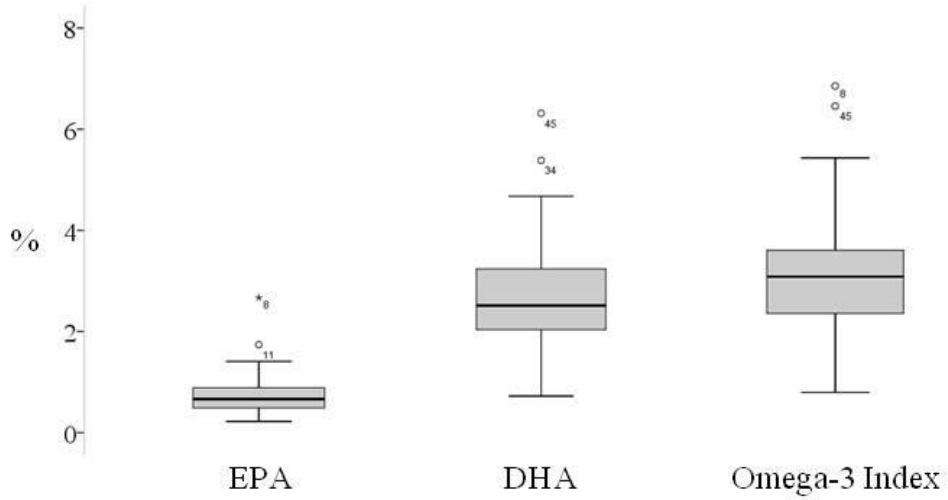
Figure 22 shows the spread of average erythrocyte membrane LC *n*-3 PUFA and O3I in the study population. The mean O3I of this population was very low (below 4%), and only 2 individuals had omega-3 indices greater than 5%. Variability in erythrocyte EPA (median [lower and upper quartile], 0.66 % [0.50, 0.89]) and DHA (mean ± SD, 2.6 % ± 0.94) was limited in this patient population, with 50% of omega-3 indices falling between 2.5% and 3.8%. and variability was lower in EPA content compared to DHA content as is observed in healthy populations, such as the MARINA study (EPA, median [lower and upper quartile], 1.16 % [1.00, 1.49] and DHA, mean ± SD, 6.6 % ± 1.42; refer to **table 20**). There was a strong significant correlation between plasma EPA and erythrocyte membrane EPA (r = 0.704; p < 0.001), and between plasma DHA and erythrocyte membrane DHA (r = 0.608; p < 0.001), which was similar to the ones seen in the age- and sex-matched healthy cohort (erythrocyte EPA and plasma EPA: r = 0.773 and p < 0.001; erythrocyte DHA and plasma DHA: r = 0.504 and p = 0.001). No correlations were found between plasma or erythrocyte LC *n*-3 PUFA proportions and estimated PUFA, EPA, DHA or oily fish intake assessed by FFQ (**Table 17**).

Table 17 - Correlations between LC *n*-3 PUFA dietary intake and plasma and erythrocyte membrane LC *n*-3 PUFA in the study population (n = 35).

Dietary intake	**Plasma EPA (%)** [a]		**Plasma DHA (%)**		**Erythrocyte EPA (%)** [a]		**Erythrocyte DHA (%)**		**Omega-3 index (%)**	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
PUFA (%)	0.264	0.131	0.050	0.779	0.247	0.159	0.045	0.800	0.125	0.481
PUFA (g)	0.083	0.642	0.018	0.918	0.038	0.831	0.045	0.802	0.023	0.897
EPA (g)	0.159	0.369	0.048	0.788	-0.125	0.483	0.142	0.422	0.057	0.749
DHA (g)	0.299	0.086	0.149	0.400	0.119	0.502	0.191	0.280	0.185	0.295
Oily fish (times per week)	0.261	0.130	0.118	0.499	0.196	0.258	0.159	0.360	0.201	0.247
Total seafood (times per week)	0.156	0.369	0.022	0.512	-0.115	0.512	0.068	0.699	0.019	0.912

[a] Correlations based on the LN variables.

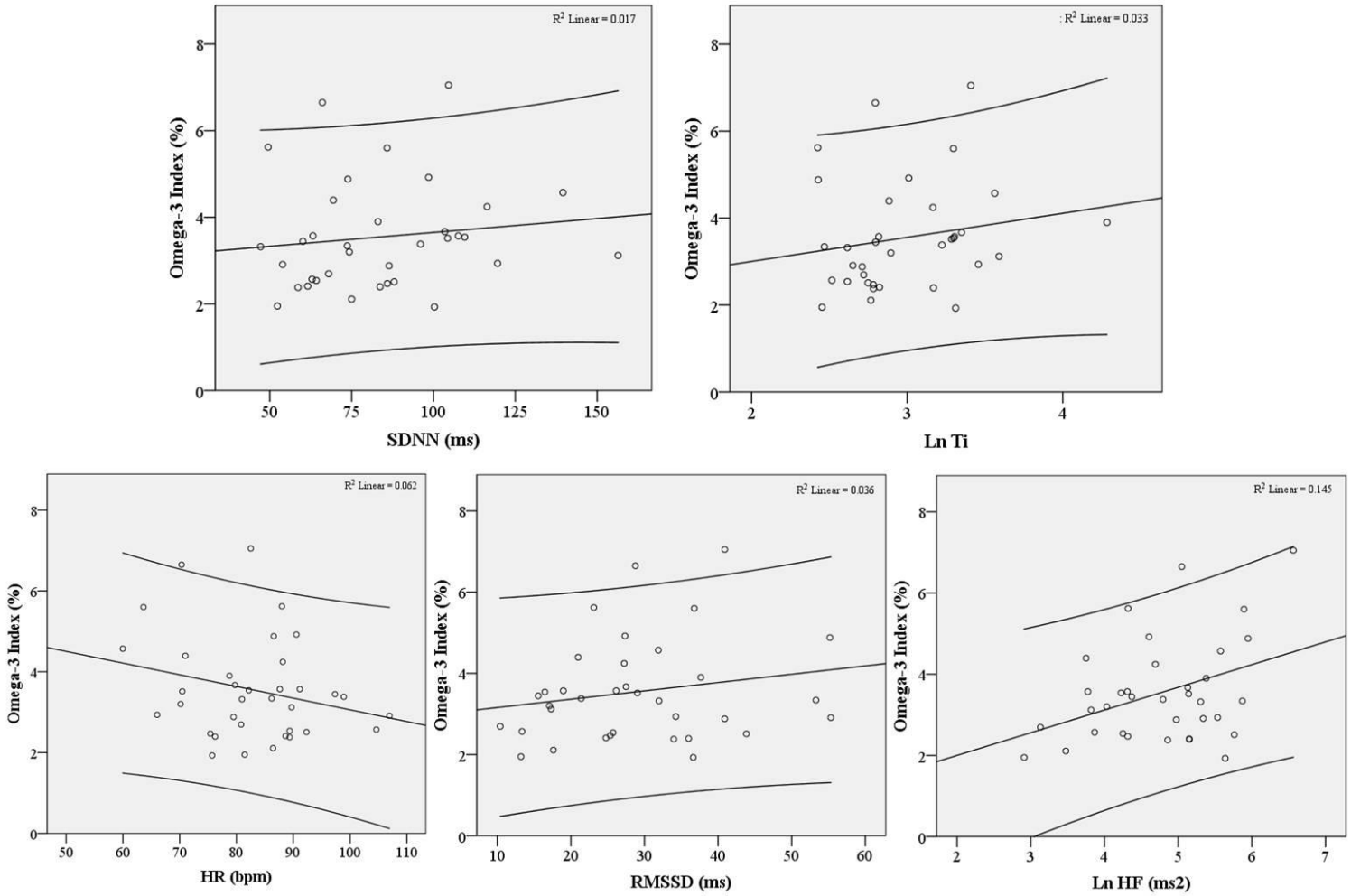
Figure 22 - Spread of erythrocyte membrane LC *n*-3 fatty acid composition in the study population.



Results presented as median (interquartile range in lower and upper box borders, with 95% confidence interval t-bars).

Figure 23 shows the correlations from the primary outcome variables, which assessed the relationship between O3I (erythrocyte membrane EPA + DHA) and 24 h SDNN and Ti. The relationship between 24 h HR and parameters reflecting vagal activity (RMSSD and HF) and O3I was also plotted. HF power correlated positively with the O3I, but no other statistically significant correlations were observed.

Figure 23 - Scatter diagrams of the relationship between erythrocyte membrane EPA+DHA (Omega-3 index) and HR (r = -0.248; p = 0.150), SDNN (r = 0.130; p = 0.456), Ti (r = 0.181; p = 0.298), RMSSD (r = 0.191; p = 0.272) and HF (r = 0.381; p = 0.024) in 24 h measurements in haemodialysis patients (n = 35).



Each figure presents linear regressions (fitted straight lines) with 95% confidence intervals (curve lines).

Table 18 further explored the relationship between HRV parameters and erythrocyte membrane O3I, as well as with EPA and DHA individually. **Table 19** presents similar comparative data for plasma EPA, DPA(*n*-3) and DHA, which were secondary outcomes of this study. Data was presented unadjusted and *p* values for 3 adjustment models were included, the first model adjusted for age alone, the second model adjusted for age, BMI and β-blocker usage (Yes/No), and the third model adjusted for age, BMI, β-blocker usage (Yes/No), diabetes (Yes/No) and hs-CRP.

Erythrocyte membrane. During dialysis the only significant correlation found was an inverse relationship between HR and EPA which remained significant after adjusting for the age but presented only a trend after further adjustments for BMI and β-blocker usage were made, and lost significance once further adjustments for diabetes and hs-CRP were made. The only relationship between HRV and LC *n*-3 PUFA found during sleep-time was a positive correlation between HF power and O3I, which remained significant after adjustment for age but lost significance after further adjustments for BMI and β-blocker usage, diabetes and hs-CRP. In the 24 h period, EPA was positively correlated with Ti, SDNN, SDANN and VLF power but after full adjustments only Ti remained statistically significant; DHA and O3I were positively correlated with LF and HF power and this association remained significant after further adjustments only for the association between LF power and DHA and kept a trend for the association between DHA and HF power, and between O3I and LF power.

Plasma. During dialysis the only significant correlation found was a positive relationship between DHA and Ti and VLF power, which remained statistically significant after full adjustment for potential known confounders. In sleep-time no significant correlations were observed after full adjustments. The more interesting results were found over 24 h measurements where EPA significantly correlated with overall and longer phase components of HRV (SDANN, VLF power and trend for SDNN) and significantly negatively correlated with beat-to-beat variability (RMSSD, pNN50 and trend for HF power) after full adjustments. In line with the correlations between EPA and beat-to-beat variability, DPA(*n*-3) was also significantly negatively correlated with 24 h HF power, with a trend for RMSSD and pNN50.

Table 18 - Correlation between erythrocyte membrane LC *n*-3 PUFA and HRV parameters during dialysis, sleep time and 24h in study participants.

	EPA [a]					**DHA**					**Omega-3 Index**				
	r	p^1	p^2	p^3	p^4	*r*	p^1	p^2	p^3	p^4	*r*	p^1	p^2	p^3	p^4
Dialysis (n=36)															
HR (bpm)	**-0.339**	**0.035**	**0.050**	0.086	0.108	-0.251	0.123	0.127	0.298	0.360	-0.344	**0.032**	**0.038**	0.124	0.159
IBI (ms)	0.204	0.212	0.237	0.582	0.677	0.187	0.253	0.260	0.302	0.527	0.230	0.160	0.172	0.255	0.482
Ti	0.229	0.160	0.351	0.582	0.682	0.231	0.157	0.137	0.056	0.126	0.263	0.106	0.126	0.060	0.158
SDNN (ms)	0.209	0.202	0.390	0.668	0.774	0.101	0.542	0.535	0.347	0.199	0.133	0.419	0.493	0.354	0.249
SDANN (ms)	0.157	0.339	0.497	0.656	0.563	0.143	0.387	0.388	0.339	0.306	0.166	0.313	0.357	0.329	0.287
pNN50 (%) [a]	0.079	0.631	0.624	0.984	0.996	0.090	0.584	0.589	0.430	0.202	0.100	0.546	0.550	0.445	0.243
RMSSD (ms)	-0.101	0.541	0.519	0.333	0.479	-0.042	0.801	0.803	0.991	0.721	-0.080	0.626	0.625	0.775	0.931
LF (ms^2) [a]	0.292	0.071	0.210	0.378	0.700	0.095	0.565	0.534	0.263	0.107	0.154	0.350	0.426	0.217	0.145
HF (ms^2) [a]	0.062	0.708	0.815	0.713	0.694	0.129	0.433	0.439	0.186	0.128	0.120	0.466	0.498	0.271	0.231
logLF:HF	0.259	0.111	0.270	0.174	0.365	-0.092	0.579	0.539	0.528	0.617	-0.013	0.937	0.775	0.800	0.809
VLF (ms^2)	0.265	0.103	0.234	0.439	0.562	0.137	0.404	0.388	0.279	0.183	0.189	0.249	0.299	0.236	0.188
SD1:SD2 [a]	-0.190	0.246	0.355	0.284	0.459	-0.029	0.863	0.868	0.993	0.850	-0.085	0.608	0.666	0.766	0.975
Sleep time (n=39)															
HR (bpm)	-0.270	0.097	0.091	0.154	0.221	-0.093	0.574	0.579	0.840	0.804	-0.169	0.304	0.310	0.522	0.840
IBI (ms)	0.258	0.113	0.113	0.182	0.292	0.081	0.625	0.631	0.850	0.804	0.154	0.349	0.360	0.532	0.869
Ti	0.297	0.067	0.091	0.429	0.638	0.059	0.722	0.732	0.701	0.933	0.173	0.293	0.327	0.879	0.976
SDNN (ms)	0.222	0.175	0.281	0.693	0.917	0.023	0.891	0.906	0.703	0.859	0.132	0.424	0.493	0.916	0.948
SDANN (ms)	0.145	0.377	0.409	0.680	0.961	-0.035	0.831	0.830	0.521	0.968	0.063	0.702	0.725	0.759	0.902
pNN50 (%) [a]	0.006	0.969	0.948	0.308	0.578	0.174	0.289	0.298	0.654	0.851	0.183	0.264	0.285	0.846	0.926
RMSSD (ms)	-0.005	0.977	0.868	0.332	0.622	0.144	0.382	0.229	0.435	0.674	0.140	0.396	0.253	0.602	0.740

	EPA [a]					DHA					Omega-3 Index				
	r	p^1	p^2	p^3	p^4	*r*	p^1	p^2	p^3	p^4	*r*	p^1	p^2	p^3	p^4
Sleep time (continued)															
LF (ms^2) [a]	0.262	0.107	0.255	0.928	0.641	0.217	0.186	0.174	0.309	0.731	0.289	0.074	0.097	0.323	0.893
HF (ms^2) [a]	0.181	0.271	0.395	0.644	0.815	0.288	0.075	0.077	0.207	0.547	0.350	**0.029**	**0.0.37**	0.247	0.544
logLF:HF	0.074	0.655	0.881	0.609	0.787	-0.118	0.474	0.460	0.674	0.648	-0.110	0.505	0.429	0.719	0.490
VLF (ms^2) [a]	0.263	0.105	0.199	0.474	0.847	-0.050	0.763	0.740	0.421	0.613	0.073	0.659	0.764	0.663	0.770
SD1:SD2 [a]	-0.171	0.299	0.351	0.194	0.568	0.209	0.202	0.204	0.267	0.513	0.127	0.440	0.415	0.515	0.659
24 h (n=35)															
HR (bpm)	-0.210	0.225	0.219	0.436	0.370	-0.211	0.223	0.239	0.430	0.744	-0.248	0.150	0.154	0.308	0.532
IBI (ms)	0.246	0.154	0.165	0.316	0.256	0.209	0.228	0.253	0.414	0.622	0.256	0.137	0.152	0.256	0.362
Ti [a]	**0.500**	**0.002**	**0.017**	**0.047**	**0.029**	0.036	0.837	0.838	0.677	0.781	0.181	0.298	0.645	0.998	0.743
SDNN (ms)	**0.381**	**0.024**	0.123	0.336	0.313	0.000	1.000	0.666	0.342	0.833	0.130	0.456	0.886	0.484	0.871
SDANN (ms)	**0.369**	**0.029**	0.130	0.271	0.249	-0.041	0.815	0.502	0.255	0.624	0.084	0.631	0.906	0.390	0.935
pNN50 (%)	-0.009	0.960	0.904	0.251	0.140	0.209	0.227	0.233	0.351	0.372	0.232	0.180	0.179	0.432	0.570
RMSSD (ms)	-0.179	0.304	0.352	0.059	0.053	0.225	0.193	0.137	0.201	0.200	0.191	0.272	0.184	0.359	0.444
LF (ms^2) [a]	0.268	0.119	0.664	0.331	0.452	**0.313**	0.067	0.105	0.145	**0.038**	**0.387**	**0.022**	0.072	0.222	0.070
HF (ms^2) [a]	0.042	0.810	0.962	0.055	0.080	**0.346**	**0.042**	**0.036**	0.068	0.071	**0.381**	**0.024**	**0.022**	0.149	0.187
logLF:HF	0.281	0.102	0.635	0.305	0.205	0.017	0.923	0.572	0.786	0.840	0.069	0.692	0.553	0.918	0.609
VLF (ms^2)	**0.382**	**0.023**	0.084	0.187	0.186	-0.025	0.886	0.632	0.377	0.846	0.102	0.561	0.913	0.588	0.776
SD1:SD2 [a]	-0.310	0.070	0.214	0.099	0.073	0.202	0.244	0.110	0.102	0.189	0.108	0.539	0.227	0.197	0.433

p^1 - unadjusted; p^2 adjusted for age; p^3 adjusted for age, BMI and β-blockers; p^4 adjusted for age, BMI, β-blockers, diabetes and hs-CRP. 24h HRV analysis has activity level from accelerometery data (cpm) as an additional adjustment for p^2, p^3 and p^4.

[a] Correlations based on LN variables

Table 19 - Correlation between plasma LC *n*-3 PUFA and HRV parameters during dialysis, sleep time and 24h in study participants.

	EPA [a]					**DPA** [a]					**DHA**				
	r	p^1	p^2	p^3	p^4	*r*	p^1	p^2	p^3	p^4	*r*	p^1	p^2	p^3	p^4
Dialysis (n=39)															
HR (bpm)	-0.173	0.292	0.328	0.614	0.761	0.125	0.448	0.400	0.433	0.253	-0.298	0.065	0.082	0.101	0.141
IBI (ms)	0.181	0.271	0.290	0.493	0.626	-0.106	0.522	0.500	0.589	0.383	0.239	0.143	0.158	0.128	0.203
Ti	0.232	0.156	0.200	0.167	0.181	0.029	0.862	0.946	0.988	0.870	**0.328**	**0.042**	0.065	**0.022**	**0.042**
SDNN (ms)	0.237	0.146	0.186	0.159	0.186	-0.003	0.983	0.814	0.879	0.671	0.252	0.122	0.178	0.081	0.100
SDANN (ms)	0.256	0.116	0.142	0.104	**0.029**	0.055	0.737	0.836	0.795	0.642	0.270	0.096	0.129	0.105	0.061
pNN50 (%) [a]	0.035	0.833	0.834	0.843	0.778	-0.090	0.584	0.588	0.637	0.564	0.056	0.737	0.737	0.567	0.530
RMSSD (ms)	-0.150	0.363	0.363	0.236	0.438	-0.213	0.193	0.193	0.196	0.342	-0.120	0.468	0.463	0.605	0.923
LF (ms^2) [a]	0.156	0.344	0.450	0.441	0.850	-0.051	0.758	0.490	0.496	0.085	0.239	0.142	0.223	0.076	0.233
HF (ms^2) [a]	0.025	0.879	0.924	0.817	0.762	-0.133	0.419	0.384	0.376	0.317	0.120	0.468	0.519	0.254	0.256
logLF:HF	0.145	0.378	0.481	0.328	0.919	0.102	0.537	0.696	0.742	0.611	0.100	0.546	0.757	0.781	0.742
VLF (ms^2)	0.314	0.051	0.066	**0.050**	0.070	0.084	0.609	0.764	0.667	0.932	**0.332**	**0.039**	0.060	**0.027**	**0.042**
SD1:SD2 [a]	-0.252	0.121	0.146	0.074	0.192	-0.213	0.192	0.232	0.244	0.514	-0.202	0.218	0.272	0.337	0.625
Sleep time (n=40)															
HR (bpm)	-0.173	0.285	0.251	0.318	0.552	0.099	0.545	0.576	0.673	0.357	-0.227	0.158	0.146	0.210	0.418
IBI (ms)	0.178	0.271	0.244	0.296	0.629	-0.077	0.636	0.661	0.778	0.356	0.216	0.180	0.170	0.218	0.502
Ti	0.250	0.119	0.161	0.282	0.447	-0.077	0.636	0.556	0.548	0.274	0.173	0.286	0.341	0.599	0.776
SDNN (ms)	0.190	0.239	0.325	0.473	0.866	0.056	0.730	0.832	0.810	0.543	0.083	0.609	0.709	0.970	0.509
SDANN (ms)	0.252	0.116	0.138	0.201	0.444	0.191	0.238	0.265	0.293	0.584	0.083	0.613	0.660	0.876	0.837
pNN50 (%) [a]	-0.191	0.237	0.223	0.095	0.303	**-0.356**	**0.024**	**0.023**	**0.023**	0.088	-0.078	0.631	0.619	0.309	0.567
RMSSD (ms)	-0.234	0.146	0.128	0.081	0.201	-0.306	0.055	0.052	0.063	0.168	-0.148	0.362	0.344	0.232	0.358

	EPA [a]					DPA [a]					DHA				
	r	p^1	p^2	p^3	p^4	*R*	p^1	p^2	p^3	p^4	*r*	p^1	p^2	p^3	p^4
Sleep time (continued)															
LF (ms^2) [a]	0.052	0.752	0.978	0.753	0.055	-0.100	0.538	0.410	0.440	**0.020**	0.107	0.511	0.637	0.823	0.336
HF (ms^2) [a]	-0.065	0.692	0.593	0.285	0.307	-0.218	0.176	0.149	0.133	0.146	0.040	0.808	0.877	0.765	0.719
logLF:HF	0.142	0.383	0.490	0.414	0.451	0.151	0.353	0.419	0.425	0.535	0.067	0.679	0.775	0.616	0.519
VLF (ms^2) [a]	0.272	0.090	0.147	0.226	0.769	0.098	0.545	0.665	0.634	0.628	0.128	0.431	0.542	0.754	0.727
SD1:SD2 [a]	-0.355	**0.025**	**0.035**	**0.026**	0.231	**-0.372**	**0.018**	**0.024**	**0.033**	0.242	-0.157	0.333	0.386	0.311	0.793
24 h (n=35)															
HR (bpm)	-0.197	0.258	0.273	0.454	0.249	0.123	0.483	0.496	0.557	0.767	-0.294	0.086	0.088	0.170	0.172
IBI (ms)	0.259	0.133	0.154	0.251	0.146	-0.078	0.658	0.656	0.751	0.875	0.328	0.055	0.061	0.103	0.110
Ti [a]	**0.369**	**0.029**	0.077	0.112	0.114	-0.164	0.345	0.241	0.260	0.068	0.306	0.073	0.222	0.244	0.185
SDNN (ms)	**0.483**	**0.003**	**0.010**	**0.025**	0.089	0.036	0.836	0.946	0.893	0.416	0.274	0.112	0.309	0.506	0.530
SDANN (ms)	**0.511**	**0.002**	**0.006**	**0.013**	**0.047**	0.068	0.698	0.781	0.734	0.587	0.260	0.131	0.337	0.525	0.544
pNN50 (%)	-0.317	0.064	0.058	**0.019**	**0.037**	**-0.378**	**0.025**	**0.028**	**0.028**	0.083	-0.028	0.873	0.833	0.636	0.654
RMSSD (ms)	**-0.395**	**0.019**	**0.023**	**0.009**	**0.015**	**-0.362**	**0.032**	**0.033**	**0.037**	0.063	-0.082	0.639	0.732	0.573	0.621
LF (ms^2) [a]	0.138	0.428	0.915	0.732	0.429	-0.055	0.755	0.508	0.525	0.184	0.259	0.132	0.462	0.574	0.602
HF (ms^2) [a]	-0.179	0.305	0.243	**0.037**	0.055	-0.293	0.088	0.073	**0.045**	**0.048**	0.098	0.575	0.655	0.986	0.918
logLF:HF	**0.371**	**0.028**	0.090	0.054	0.137	0.269	0.119	0.119	0.125	0.324	0.223	0.197	0.676	0.481	0.557
VLF (ms^2)	**0.503**	**0.002**	**0.006**	**0.013**	**0.043**	0.038	0.827	0.912	0.850	0.536	0.262	0.129	0.285	0.422	0.445
SD1:SD2 [a]	**-0.574**	**<0.001**	**0.001**	**0.001**	**0.004**	**-0.363**	**0.032**	**0.038**	**0.045**	0.168	-0.212	0.221	0.451	0.453	0.499

p^1 - unadjusted; p^2 adjusted for age; p^3 adjusted for age, BMI and β-blockers; p^4 adjusted for age, BMI, β-blockers, diabetes and hs-CRP. 24h HRV analysis has activity level from accelerometery data (cpm) as an additional adjustment for p^2, p^3 and p^4.

[a] Correlations based on LN variables

The low spread of LC *n*-3 PUFA erythrocyte levels in this study population prevented grouping the patients according to a low, medium and high O3I to further explore the relationship between HRV and LC *n*-3 PUFA tissue status. For this reason, comparisons with matched healthy participants from the MARINA study (see **Chapter 3**, page 79 for study details) were made for sleep time HRV. **Table 20** shows the comparison of haemodialysis patients with a subgroup of age- and sex-matched healthy participants from the MARINA study. Compared to the healthy cohort, haemodialysis patients showed significantly higher HR and lower HRV during sleep as well as significantly lower proportions of erythrocyte membrane LC *n*-3 PUFA, while not reporting significantly different EPA and DHA dietary intakes.

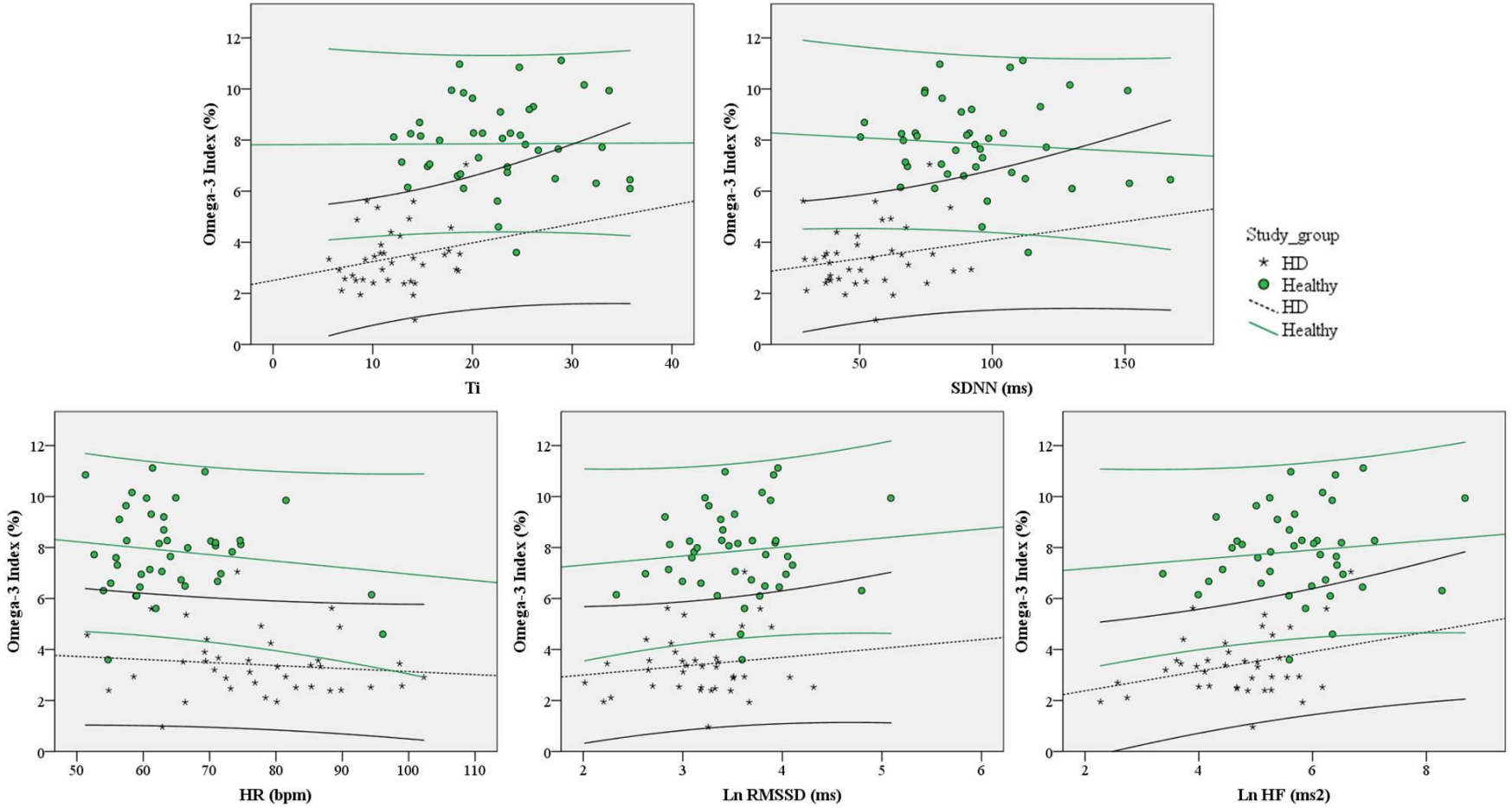
Table 20 - Inflammatory marker, LC *n*-3 PUFA tissue status and HRV during sleep time in haemodialysis patients compared to age- and sex-matched healthy controls from the MARINA study.

	Haemodialysis cohort (n = 42)	**Healthy cohort (n = 42)**	***p* value** [†]
Sex (M:F)	22 : 20		
Age (y)	57 ± 8		
Ethnicity (White:Black:Other)	19 : 18 : 5	32 : 7 : 3	**0.013** [§]
BMI (kg/m^2)	27.6 ± 7.0	26.1 ± 3.4	0.231
hs-CRP (mg/L) [a]	5.0 (3.3, 7.6)	0.6 (0.4, 0.9)	**<0.001**
EPA dietary intake (mg/day)	0.09 (0.06, 0.14)	0.10 (0.07, 0.13)	0.732
DHA dietary intake (mg/day)	0.27 (0.16, 0.45)	0.18 (0.13, 0.25)	0.176
Erythrocyte EPA (%) [a]	0.6 (0.54, 0.77)	1.2 (1.09, 1.35)	**<0.001**
Erythrocyte DPA (%) [a]	1.5 (1.37, 1.63)	3.3 (3.1, 3.5)	**<0.001**
Erythrocyte DHA (%)	2.6 ± 0.94	6.6 ± 1.42	**<0.001**
Omega-3 index (%)	3.4 ± 1.17	7.9 ± 1.67	**<0.001**
Plasma EPA (%) [a]	0.8 (0.63, 0.91)	1.0 (0.91, 1.18)	**0.007**
Plasma DPA (%) [a]	0.44 (0.40, 0.47)	0.64 (0.60, 0.68)	**<0.001**
Plasma DHA (%)	1.8 ± 0.65	2.4 ± 0.63	**<0.001**
HR and HRV parameters	N = 40	N = 40	
Sleep time			
HR (bpm)	77.4 ± 12.1	64.6 ± 9.7	**<0.001**
IBI (ms)	800 ± 133	962 ± 115	**<0.001**
Ti	12.2 ± 3.8	22.6 ± 6.3	**<0.001**
SDNN (ms)	53.1 ± 16.7	94.3 ± 25.9	**<0.001**
pNN50 (%) [a]	2.6 (1.7, 4.1)	6.2 (4.1, 9.5)	**0.006**
RMSSD (ms) [a]	23.5 (19.9, 27.8)	34.4 (29.4, 40.4)	**0.001**
LF (ms^2) [a]	104 (79, 137)	655 (504, 852)	**<0.001**
HF (ms^2) [a]	106 (76, 148)	316 (231, 432)	**<0.001**
logLF:HF	-0.01 ± 0.82	2.64 ± 1.91	**<0.001**
VLF (ms^2) [a]	1514 (1257, 1824)	4932 (4132, 5888)	**<0.001**
SD1:SD2 [a]	0.24 (0.21, 0.29)	0.20 (0.17, 0.23)	0.073

BMI, body mass index; DBP, diastolic blood pressure; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; HR, heart rate; PUFA, polyunsaturated fatty acids; SBP, systolic blood pressure. Results expressed as a ratio or mean ± SD, except [a] Geometric means (95%CI) and [†] *p* value obtained by independent t-test, except [§] χ^2 test.

Figure 24 shows the correlations between the primary outcome variables during sleep-time in the haemodialysis patients and the healthy cohort. There were no significant correlations within each group. There was only a trend for a positive relationship between O3I and HF in the haemodialysis group but after further adjustments there was no longer statistical significance.

Figure 24 - Scatter diagrams of the relationship between erythrocyte membrane EPA + DHA (Omega-3 index) and Ti (r = 226; p = 172 vs. r = 0.006; p = 0.969), SDNN (r = 0.199; p = 0.230 vs. r = -0.084; p = 0.595), HR (r = -0.118; p = 0.481 vs. r = -0.147; p = 0.352), RMSSD (r = 0.146; p = 0.383 vs. r = 0.111; p = 0.484) and HF (r = 0.313; p = 0.056 vs. r = 0.115; p = 0.469) during sleep-time in haemodialysis patients (n = 40) and age- and sex-match healthy cohort (n = 40) respectively.



Each figure presents linear regressions (fitted straight lines) for each group with respective 95% confidence intervals (curve lines).

In order to assess how the HRV in this population of patients compares with similar patient populations reported previously, **table 21** and **table 22** show HRV during dialysis and 24h, respectively, in the current population (FISHH study, UK) with other published studies. Compared to other studies measuring HRV during dialysis, the FISHH study presented HR values similar to the ones reported in the other UK study population during the first hour of dialysis (263) and similar to the group that presented ischaemic lesions in the French study population (264). Overall, the HRV across the studies was depressed, particularly the frequency-domain indices, compared to healthy populations. The FISHH study presented lower HRV during dialysis, although better LF : HF ratio in both dialysis and 24 h, and lower IBI and overall HRV (SDNN and SDANN) but higher 24 h beat-to-beat variability (pNN50, RMSSD and HF power).

Table 21 - Comparison of the present study (FISHH study) with other published studies on HRV parameters measured during haemodialysis in stage 5 CKD patients.

HRV parameters	**FISHH study**	**Tong** (265)	**Mann** (266)	**Poulikakos** (263)		**Coquet** (264)	
	UK	**China**	**Canada**	**UK**		**France**	
	N = 39	N = 35	N = 56	N = 76		N = 18	N = 14
				1st h on HD	Last h on HD	No ischaemia group	Ischaemia lesions group
HR (bpm)	77.2 ± 15.0			77 ± 11	80 ± 14	71 ± 10	75 ± 9
IBI (ms)	787 ± 131						
Ti	11.0 ± 3.6						
SDNN (ms)	45.8 ± 13.9	36.3 ± 10.6	88 ± 13				
SDANN (ms)	32.7 ± 12.6		72 ± 14				
pNN50 (%)	1.7 (1.0, 2.8) [a]	27.6 ± 2.3	9.2 ± 2.5			3.8 ± 5.2	2.6 ± 4.7
RMSSD (ms)	22.5 (18.4, 27.4) [a]	22.3 ± 6.2				24.5 ± 18	22.2 ± 15
LF (ms^2)	89 (62, 128) [a]	53 ± 21	586 ± 108	[x]	[x]	107 *	81 *
HF (ms^2)	97 (67, 142) [a]	53 ± 11	312 ± 62	[x]	[x]	48 *	38 *
LF:HF	0.98 (0.85, 1.13) [a]	1.02 ± 0.85	1.40 ± 0.08			3.40 ± 2.66	2.70 ± 2.25
logLF:HF	-0.02 ± 0.43			0.39 ± 0.27	0.43 ± 0.33		
VLF (ms^2)	1338 ± 821						
SD1:SD2	0.27 (0.23, 0.33) [a]						

Results expressed as mean ± SD, except [a] Geometric means (95% CI) [x] Values measured in Hz so these are not comparable. *Values were unlogged from the paper to allow for comparison, therefore SD is unobtainable.

Table 22 - Comparison of the present study (FISHH study) with other published studies on 24h HRV parameters in stage 5 CKD patients undergoing haemodialysis.

Study	**FISHH study**	**Svensson** (111)	**Chan** (267)	**Oikawa** (255)		**Fukuta** (253)		
Country	**UK**	**Denmark**	**USA**	**China / Japan**		**Japan**		
	N = 35	N = 15	N = 239	N = 237	N = 146	N = 99	N = 10	N = 11
				Survivor	Non-survivor	Survivor	Cardiac death	Non-cardiac death
HR (bpm)	83.0 ± 10.9	73.8 ± 8.5						
IBI (ms)	747 ± 102	824 ± 102	812 ± 217	777 ± 97	775 ± 105	748 ± 98	718 ± 120	750 ± 57
Ti	19.7 (17.1, 22.8) [a]					25.4 ± 8.9	17.9 ± 6.2	30.2 ± 13.8
SDNN (ms)	84.1 ± 25.6	100 ± 45	79.1 ± 40.3	111 ± 33	85 ± 34	96.8 ± 32.3	77.5 ± 35.0	104.7 ± 48.1
SDANN (ms)	76.2 ± 25.6	90 ± 45		103 ± 32	80 ± 32			
pNN50 (%)	4.8 ± 4.0			3.16 ± 5.96	2.19 ± 4.86			
RMSSD (ms)	29.0 ± 11.7	21.2 ± 14.6		19 ± 11	17 ± 9			
LF (ms^2)	95 (68, 132) [a]		106 (48.0, 204) [b]	119 *	42 *	113 *	57 *	107 *
HF (ms^2)	118 (88, 158) [a]		42.4 (29.4, 56.3) [b]	38 *	26 *	81 *	90 *	97 *
LF:HF	0.80 (0.61, 1.05) [a]		2.37 (1.43, 3.93)	1.33 ± 0.25	1.17 ± 0.29	1.82 ± 1.34	0.77 ± 0.44	1.60 ± 1.81
LogLF:HF	-0.22 ± 0.79							
VLF (ms^2)	4844 ± 3218					503*	209*	469*
SD1:SD2	0.17 (0.14, 0.20) [a]							

Results expressed as mean ± SD, except [a] Geometric means (95% CI) and [b] median (IQR) *Values were unlogged from the paper to allow for comparison, therefore SD is unobtained.

Table 22 (continued) - Comparison of the present study (FISHH study) with other published studies on 24h HRV parameters in stage 5 CKD patients undergoing haemodialysis.

Study	**FISHH study**	**Christensen** (106)	**Coquet** (264)	
Country	**UK**	**Denmark**	**France**	
	N = 35	N = 29	N = 18	N = 14
			No coronary ischaemia group	Coronary ischaemia group
HR (bpm)	83.0 ± 10.9		74 ± 11	77 ± 10
IBI (ms)	747 ± 102	752 ± 131		
Ti	19.7 (17.1, 22.8) [a]			
SDNN (ms)	84.1 ± 25.6	86 ± 31		
SDANN (ms)	76.2 ± 25.6			
pNN50 (%)	4.8 ± 4.0		4.5 ± 2.7	1.7 ± 1.4
RMSSD (ms)	29.0 ± 11.7		27.7 ± 13.4	19.7 ± 6.8
LF (ms^2)	95 (68, 132) [a]		133 *	73 *
HF (ms^2)	118 (88, 158) [a]		65 *	38 *
LF:HF	0.80 (0.61, 1.05) [a]		3.37 ± 2.61	2.85 ± 2.61
LogLF:HF	-0.22 ± 0.79			
VLF (ms^2)	4844 ± 3218			
SD1:SD2	0.17 (0.14, 0.20) [a]			

Table 23 presents similar comparative data for erythrocyte and serum/plasma LC *n*-3 PUFA composition. The present study (FISHH, UK), presents the lowest erythrocyte membrane DHA proportions, which translated in the lowest O3I, compared to the other studies that reported comparable results in haemodialysis patients. The studies in Japanese and Korean populations presented particularly high proportions of EPA + DHA. Plasma proportions of EPA + DHA was more homogeneous across the studies, although the FISHH study had EPA and DHA proportions amongst the lowest compared to the other haemodialysis populations, except for the USA study populations.

Table 23 - Comparison of the present study (FISHH study) with other published studies on erythrocyte membrane and plasma/serum LC *n*-3 PUFA levels in stage 5 CKD patients.

	FISHH study **N=45** **UK**	**Hamazaki** (268) **N = 176** **Japan**	**Hamazaki** (269) **N = 12** **Japan**	**Oh** (270) **N = 68** **Korea**	**An** (271) **N=15** **Korea**	**Ristić** (272) **N=37** **Serbia**	**Koorts** (273) **N = 14** **South Africa**
Erythrocyte membrane °							
20:5n-3, EPA (%)	0.65 (0.54, 0.76) [a]	2.0 ± 1.0		1.98 ± 1.03	3.03 ± 1.92	0.23 ± 0.06	0.20 ± 0.07
22:6n-3, DHA (%)	2.51 (2.22, 2.83) [a]	7.7 ± 1.1		6.62 ± 2.20	10.03 ± 1.68	4.33 ± 0.83	3.74 ± 0.81
Omega-3 Index (%)	3.24 (2.90, 3.62) [a]			8.59 ± 2.98	13.07 ±3.02		
Serum / Plasma							
20:5n-3, EPA (%)	0.75 (0.32, 0.91) [a]		1.2 ± 0.7			0.26 ± 0.14	
22:6n-3, DHA (%)	1.78 ± 0.62		1.9 ± 0.6			3.00 ± 0.90	

Results expressed as mean ± SD, except [a] Geometric means (95% CI). ° N=44 due to technical issue with one sample

Table 23 (continued) - Comparison of this study (FISHH study) with other published studies on erythrocyte membrane and plasma/serum LC *n*-3 PUFA levels in stage 5 CKD patients.

	FISHH study	**Saifullah** (274) **N = 23** **USA**	**Peck** (275) **N= 25** **USA**	**Friedman** (276) **N = 75** **USA**	**Svensson** (111) **N=15** **Denmark**	**Madsen** (246)***** **N= 44** **Denmark**
Erythrocyte membrane °						
20:5n-3, EPA (%)	0.65 (0.54, 0.76) [a]	0.3 ± 0.2		0.29 ± 0.08		
22:6n-3, DHA (%)	2.51 (2.22, 2.83) [a]	2.9 ± 2.0		4.65 ± 0.92		
Omega-3 Index (%)	3.24 (2.90, 3.62) [a]			4.95 ± 0.95		
Serum / Plasma						
20:5n-3, EPA (%)	0.75 (0.32, 0.91) [a]	0.5 ± 0.4	0.46 ± 0.45	0.35 ± 0.32	1.3 ± 0.6	1.58 ± 1.35
22:6n-3, DHA (%)	1.78 ± 0.62	1.7 ± 0.6	1.59 ± 0.55	1.33 ± 0.38	4.3 ± 0.8	4.07 ± 1.35

Results expressed as mean ± SD, except [a] Geometric means (95% CI). * Index study (2009) from Madsen et al. ° N=44 due to technical issue with one sample

5.8 Discussion

This study is the first to characterise the variability of erythrocyte membrane LC *n*-3 PUFA in patients who have recently commenced haemodialysis as well as exploring relationships with HRV parameters. Moreover, this is also the first known study to date to determine LC *n*-3 PUFA levels in haemodialysis patients in a UK-based population. Haemodialysis patients in other countries have been shown to have lower EPA, DHA, or both EPA + DHA, in erythrocyte membranes and / or plasma compared to healthy controls or healthy populations (246,272,275,277,278). Only one study by Friedman et al. showed a greater erythrocyte DHA and O3I, but lower plasma DHA, in haemodialysis patients (n=75) compared to a healthy control (n=25) (276). Compared to other haemodialysis cohorts, the mean levels of DHA and O3I in the present study population were amongst the lowest, and were markedly lower than an age- and sex-matched healthy cohort.

Erythrocyte membrane fatty acid composition is considered to be a better marker of cardiovascular protection compared to plasma, as it has been shown to be more reflective of the cardiomyocytes fatty acid composition (174). None of the patients in this study presented an O3I above 8% which has been associated with the greatest cardio-protection and the mean O3Imega-3 index was below 4%, which has been associated with the least cardio-protection (205). This may reflect background diet to some degree, although this was not supported by food frequency questionnaire data on self-reported fish intake, or it may be due to the high level of uraemia presented right before dialysis as the uraemic nature of the blood samples posed an additional challenge to determine the erythrocyte membrane fatty acid composition. It is most likely that the major contributing factor to low LC *n*-3 PUFA content in erythrocytes is related to the pathology of kidney disease. Anaemia is prevalent in stage 5 CKD mainly due to low production of erythropoietin, a hormone produced by the kidney that regulates erythrocyte production from the bone marrow (necessitating erythropoietin therapy in most haemodialysis patients), and also as a result of uraemic inhibitors of erythrocyte production (279). Other factors include reduced lifespan of erythrocytes due to oxidative stress, inflammation and haemolysis, which may be a limitation in using erythrocyte membrane fatty acid composition as

a marker of general tissue membrane fatty acid status (cardiac myocytes, neurons, etc). Thus, although the utility of erythrocyte fatty acid composition as a biomarker of moderate-long term dietary intake has been established in other populations (280), the same relationships with dietary intake and tissue status cannot be assumed in populations in the advanced stages of CKD. These relationships are likely to be further disrupted in patients receiving haemodialysis due to haemodialysis-related factors such as chronic inflammation, oxidative stress and altered lipid metabolism/uptake into membranes. Our data show that variability in erythrocyte LC *n*-3 PUFA contents is indeed limited to a small range, and it is unknown whether this is an accurate representation of general tissue status.

The main findings from this study were that the primary HRV outcomes, 24 h SDNN and Ti, did not correlate with O3I and further analysis didn't find any correlations between O3I and other HRV parameters in dialysis, sleep-time or 24 h. The only correlations that were still significant after all adjustments for potential confounders were between 24 h LF power and DHA, and between Ti and EPA which due to the amount of correlations performed could be a type I error. The lack of relationship between HRV and LC *n*-3 PUFA in this study could also be due to the limited variability of erythrocyte membrane EPA, DHA and O3I in this population. For this reason, it seems unlikely that the erythrocyte membrane O3I would be a good marker for LC *n*-3 PUFA content of other tissues relevant to cardiac autonomic function, such as the heart and neural tissue in patients on haemodialysis. Previously, there were no published studies correlating frequency-domain HRV parameters with LC *n*-3 PUFA. Only two published studies to date assessed the relationship between LC *n*-3 PUFA and time-domain HRV in stage 5 CKD patients on dialysis; Christensen et al found positive correlations between granulocyte EPA, DHA and total LC *n*-3 PUFA content and 24 h SDNN in 17 patients on haemodialysis or peritoneal dialysis after a fish-oil vs. olive oil dietary intervention (106), and Svensson et al measured LC *n*-3 PUFA in serum phospholipids in 30 patients and found no correlations with HRV measurements at baseline (111). Madsen et al. has conducted a RCT, placebo-controlled, double blind study investigating the effect of LC *n*-3 PUFA infusion on plasma and platelet phospholipids fatty acid composition and HRV but to date only the data on LC *n*-3 PUFA was published (281). Plasma EPA significantly positively correlated with overall

and longer phase components of HRV (SDANN, VLF power and trend for SDNN) and significantly negatively correlated with beat-to-beat variability (RMSSD, pNN50 and trend for HF power) over 24 h after full adjustment for main potential known confounders. As there were no correlations with 24 h DHA, the fact that increases in EPA significantly correlate with decreases in beat-to-beat variability may suggest that these patients could have a limit degree of conversion of EPA to DHA. Further analysis revealed that DPA(*n*-3), the next fatty acid in the interconversion cascade from EPA to DHA, was significantly negatively correlated with the same beat-to-beat HRV parameters (HF power and trend for pNN50 and RMSSD), but had no correlations with the longer-phase components. This indicates that a greater EPA (but not DPA or DHA) proportion in plasma, is associated with better overall and longer-phase autonomic regulation, generating the hypothesis that greater availability of EPA-derived lipid mediators may be involved in increased responsivity of HR to factors such as circadian fluctuations in hormone secretion and baroreflex. However, the beat-to-beat-regulation seems to be negatively affected by greater proportions of EPA, and the same trend for DPA, which suggests that a compromised conversion of DPA to DHA might reduce parasympathetic tone due to reduced availability of DHA in the membranes of cardiomyocytes and/or a reduction in DHA-derived lipid mediators involved in neuroprotective processes in this patient population.

Patients in the FISHH study had SDNN measurements amongst the lowest in comparison to other HD cohorts. This could be partly explained by duration of HD treatments being that these were patients that recently started HD (measurements taken between 6 and 10 weeks of commencing treatment). Although commencing HD is associated with autonomic dysfunction, Tamura et al. reported the lowest SDNN levels in patients on HD for less than 30 months compared to those above 30 months (282). Moreover, after the start of HD treatments autonomic dysfunction of patients has been reported to improve or remain unaltered compared to patients with chronic renal failure before starting HD (283). Fukuta et al also showed that decreases in Ti independently predicted cardiac death (253) and Hayano et al. reported an independent prognostic value of Ti (<22) to predict both all-cause and sudden death (hazard ratio (95% CI); 8.1 (1.3, 48.6) and 12.6 (1.3, 126.4), respectively) (254). In the present study 22 patients presented Ti < 22 ms which corresponds to 65% of those with valid 24h HRV readings.

Hayano et al also reported a 24 h SDNN below 50 ms to predict a greater risk of sudden death in 31 chronic haemodialysis patients with stage 5 CKD after 5 years of follow-up (254). Although only 2 patients presented SDNN under 50 ms in the present study, the mean 24 h SDNN of this study population was 84 ms which would be considered to be a moderately depressed HRV. This finding is consistent with other published studies reporting depressed HRV in haemodialysis patients (**Table 22**). In addition, effects of medication on HRV should be considered in this poly-medicated population. Treatment with β-blockers has been shown to increase HRV (284,285) and splitting the patients with valid 24 h HRV recordings into users (n=16) and non-users (n=18) of β-blockers showed that those taking β-blockers had a statistically significant lower HR (78 vs. 87, $p = 0.017$) and higher IBI (795 vs. 705, $p = 0.009$) and HF (161 vs. 81, $p = 0.011$). This agrees with a RCT in 13 patients with stage 5 CKD on haemodialysis where HF significantly increase after intravenous administration of propranolol (286).

HRV is substantially reduced in haemodialysis patients compared to healthy controls as shown by previous studies comparing haemodialysis patients to age matched healthy controls and stage 5 CKD has been suggested to be an independent factor for a decreased HRV (287). This study confirms and extends these findings demonstrating consistently that time-domain and frequency-domain HRV parameters were significantly lower in haemodialysis patients compared to age- and sex-matched healthy controls from the MARINA study. Other studies reporting reduced HRV in stage 5 CKD patients compared to healthy participants (282) have not age- and sex-matched the healthy cohort and have not studied as extensively the different HRV parameters as the comparison done in this chapter. CRP has been previously positively associated with mortality in haemodialysis patients (288). The haemodialysis patients in this study presented significantly higher hs-CRP values compared to the healthy cohort, showing that inflammation could be a possible factor contributing for the lower LC *n*-3 PUFA and HRV levels compared to healthy individuals. There were no significant correlations between hs-CRP and HRV measurements in this study population after adjusting for age and BMI, which could be due to the small sample size. One study reported an inverse relationship between IL-6 and SDNN, SDANN and VLF in CKD patients but in dialysis patients in the same study, no

associations between HRV and inflammatory markers were found (93). Studies with a larger sample size showed independent associations between HRV and CRP in healthy and in patients with CHD (289) and a meta-analysis showed that CRP predicted CHD independent of traditional cardiovascular risk factors (290).

This study presents several limitations, including multiple correlation tests because of the exploratory nature of the study, which might have generated significant associations that may have been type I errors. The sample size of 70 to 80 participants was not achieved due to multiple unforeseen challenges in the participant recruitment, compliance and retention of patients for the second visit. These unforeseen challenges related to obstacles faced by a non-clinical researcher (working outside the NHS) recruiting at multiple NHS sites across London, (which is not feasible for a single researcher to work across 10 sites) relying on the goodwill of NHS employees to facilitate the study, and also the goodwill of patients who were already undergoing a highly invasive therapy and therefore may have been reluctant to undergo any additional procedures, however non-intrusive they may be. In addition, the patients enrolled in this study had different treatment schedules with patients dialysing at different times of the day (morning, afternoon or evening) which may have induced circadian differences in the readings. Another limitation of this study is the number of comorbidities presented by most patients which could have affected independently the HRV measurements not allowing the detection of potential associations with LC *n*-3 PUFA status.

Patients with stage 5 CKD are at high risk of SCD and there are numerous mechanisms that could be involved in the electrophysiological instability that predispose these patients to malignant cardiac arrhythmias. These patients have a moderate to severe cardiac autonomic dysfunction and this may be a reason for the increased risk of cardiac events and death. This study population presented a particularly low HRV and LC *n*-3 PUFA levels compared to other haemodialysis patients and even more when compared to healthy cohorts together with levels of CRP indicative of a chronic low-grade inflammation. For this reason, this population would be a suitable candidate to test the cardiovascular benefits of LC *n*-3 PUFA supplementation. In order to justify such a clinical trial, data are needed showing associations between LC *n*-3 PUFA tissue status and HRV in patients in advanced stages of CKD. The main finding from the current

feasibility study is that erythrocyte LC *n*-3 PUFA are very low in patients who have recently commenced haemodialysis, and do not vary substantially within the group, which precludes any meaningful correlation analysis within this population. It can be concluded that the current study design should not be extended to a larger cross-sectional study of the same design, and that other tissue biomarkers of LC *n*-3 PUFA status should be explored in feasibility studies, such as platelets and mononuclear cells, which have been suggested as the most suitable biomarker of habitual LC *n*-3 PUFA intake (291).

In summary, haemodialysis patients presented particularly low proportions of erythrocyte membrane EPA + DHA, which precluded any meaningful findings in the study primary outcome - the relationship between tissue LC *n*-3 PUFA status and HRV. However, plasma LC *n*-3 PUFA analysis revealed that there may be a compromised conversion of DPA to DHA in patients that recently started haemodialysis which is negatively impacting the vagally-mediated regulation of cardiac autonomic function, and this could be a potential mechanism whereby these patients present with greater risk of SCD.

Chapter 6 Comparison of the effects of intermittent vs. continuous energy restriction diets on heart rate variability: a randomised controlled trial

6.1 Introduction

Obesity is a risk factor for the development of CVD and has been shown to alter ANS activity, presenting a higher risk of SCD. Chronic overactivation of the SNS, which is reflected by a low HRV, is thought to be a key factor in the pathogenesis of obesity-induced endothelial dysfunction and hypertension (292,293). A dysfunctional ANS carries consequences for obese subjects, namely reduced cardiovascular resilience and adaptability to daily activity demands and stress that increases their risk for cardiac events.

Weight loss has been shown to improve HRV in obese subjects and carries cardio-metabolic benefits (refer to **Chapter 1, section 1.5.1.2**). The traditional approach to weight loss is based on a continuous energy restriction (CER) which involves a reduced daily energy intake relative to total energy expenditure in order to promote a negative energy balance to achieve moderate weight loss. Intermittent energy restriction (IER) is an alternative weight loss approach that consists of a predefined period of time severely restricting energy intake, alternated with a period of greater energy intake (294). In animal models, imposing fasting periods has been shown to confer metabolic advantages over reducing daily energy intake (295) but seems to have comparable effects on improving ANS activity (296). Fasting may affect ANS activity via β-hydroxybutyrate antagonistic effects on FFA3 receptors, which regulate energy balance by modulating sympathetic activity. Under fasting conditions, β-hydroxybutyrate inhibits FFA3 receptors in sympathetic ganglion, suppressing sympathetic nerve activity (297). A previous pilot study (The LighterHeart Study, unpublished data)[8] comparing the short-term adherence (1 week) to a very low calorie diet (VLCD, 600 kcal per day) with a moderate energy restriction (MER) diet in obese subjects showed a significant increase in HF power after the VLCD compared to an overall decrease following a MER diet, indicating increased parasympathetic regulation. Since these results were obtained with minimal

[8] Conference presentation, oral short communication, Glasgow, UKCO, 2016

weight loss, the increase in vagal tone might be only indirectly related to the weight loss itself. However, few human trials have studied the effects of IER directly comparing with an isoenergetic CER control group. Only a couple of published studies to date compared the two dietary approaches and showed that following an IER diet conferred additional metabolic benefits (increased insulin sensitivity) in overweight and obese women (298,299) but the effects of following an IER diet on HRV in humans is unknown. Increases in fat mass, particularly visceral fat, contribute to adipose tissue dysfunction and increased risk of cardio-metabolic disease through an underlying low grade chronic inflammation, resulting in altered adipokine production and insulin resistance (292). Abdominal visceral fat has been strongly associated with SNS activation (35) and high reactivity of the sympathetic vs. parasympathetic nervous system has been proposed as a link between central obesity and development of insulin resistance and type 2 diabetes (300). It has also been suggested that stress-induced sympathetic overactivity may promote visceral fat accumulation (301).

Preliminary data from the LighterHeart Study showed that favourable changes in ANS function occur rapidly following onset of severe energy restriction (SER, within 1 week) compared to MER. Therefore, there is the possibility of some other mechanism, independent of weight loss, that might have additional benefits on the PNS/SNS activity but it is unknown whether there would be further changes over a longer period of time. The present research trial investigates the short-term effects (over 1 month) of an IER diet with two consecutive days of SER (600 kcal per day, considered a modified fasting) on HRV, insulin sensitivity and inflammation compared with an isoenergetic CER diet, aiming at the same amount of weight loss, in centrally obese but otherwise healthy adults. The mechanistic effects of any additional benefits for the cardiac autonomic control conferred by losing weight via an IER diet vs CER diet will also be explored.

6.2 Hypothesis

It is hypothesised that centrally obese adults will have greater reductions in HR and greater increase in HRV parameters following IER compared to CER. Furthermore, these

differences between groups will be associated with differences in markers of inflammation, insulin sensitivity, and blood pressure, and be independent of weight loss.

6.3 Aims

This study aims to assess the impact of an IER diet compared to an isoenergetic CER diet on HRV, insulin sensitivity, inflammatory markers, and blood pressure.

6.4 Methods

This study was funded by LighterLife® (UK) Ltd.

6.4.1 Study design

This was a 4-week parallel arm RCT designed to compare the relative effects of short-term IER and CER diets on cardio-metabolic risk factors in centrally obese men and women. Healthy adults, with waist circumference measurements exceeding ethnic group-specific thresholds for substantially increased risk of cardio-metabolic disease were recruited and randomly allocated to 1 of 2 treatments. The control arm was a standard CER diet where subjects were provided with dietary advice aimed at reducing daily energy intake by 500 kcal relative to their estimated total energy expenditure; a weekly deficit of 3500 kcal. The energy-matched experimental arm involved SER for 2 consecutive days (2-d SER) of the week (~600 kcal per day) and the other 5 days of the week on a standard energy or MER diet calculated to be equivalent to a weekly deficit of 3500 kcal relative to estimated total energy requirements.

6.4.2 Ethics and consent

Ethical approval was obtained from KCL Research Ethics Committee (REC) (ref: HR-15/16-2179) and the study was registered at www.clinicaltrials.gov (ref: NCT02679989). The study was conducted in the MRU at Franklin-Wilkins Building, KCL between February and July 2016. Participants gave written informed consent before participation and received a small remuneration for taking part.

6.4.3 Outcome measures

The primary clinical outcome of the Met-IER study was differences between treatments in R-QUICKI, an index of insulin sensitivity based on fasting blood concentrations of glucose, insulin and non-esterified fatty acids (NEFA). The primary mechanistic outcome for HRV was differences between treatments in HF power, RMSSD, and pNN50, indicators of parasympathetic activity (vagal tone). Reduced HRV has previously been associated with insulin resistance in non-diabetic individuals (302,303).

Secondary outcomes include other indicators of ANS function, including time and frequency-domain and non-linear parameters of HRV, and additionally ABP, since the ANS is a significant contributor to the long-term regulation of blood pressure (304). Plasma catecholamine markers were also measured as indicators of sympathetic activity (metanephrine, a stable metabolite of epinephrine, and normetanephrine, a stable metabolite of norepinephrine); norepinephrine is continuously released into the circulation at low levels from synapses as "spillover" from sympathetic neurotransmission, therefore changes in normetanephrine might indicate changes in sympathetic nerve activity (305). Measurements of body composition (waist and hip circumference, BMI, percentage body fat) were made to monitor the impact of the energy restriction regimes on fat distribution. Full lipid profiles (total, LDL and HDL cholesterol, triglycerides, total cholesterol : HDL cholesterol ratio) were measured as an additional marker of CVD risk. Adiponectin and leptin were analysed to determine differential responses in adipose tissue function to CER and IER, and IL-6 was measured to indicate any differences in inflammatory status following the diets. Other secondary outcomes that were not included in the results of this doctoral research were digital volume pulse, eating behaviour/emotion/mood questionnaires (e.g. Three Factor Eating Questionnaire revised 18 items version 2 (TFEQR18V2), COPE, Power of Food Scale), faecal sample (for microbial composition analysis) and cognitive function (Mnemonic Similarity Test).

Body weight and β-hydroxybutyrate were outcome variables that indicated compliance to diet and fasting, respectively.

6.4.4 Power calculation, inclusion and exclusion criteria

The power calculation for this study was based on previously published data (223) and performed for the primary outcome of this study (R-QUICKI). In order to detect a mean difference between groups of 0.060 (arbitrary units) with 80% power and an alpha of 0.05, 17 participants per arm were required. The aim was to recruit 46 subjects in total to allow for 25% drop-out rates.

Based on a previous study by our group (LighterHeart Study) that detected a difference of 164 $msec^2$ (SD 180) in HF power between a 1-week VLCD compared to a MER diet (unpublished data), a sample size of 17 participants in each group has 80% power to detect mean changes in HF power of 179 $msec^2$ with an alpha of 0.05.

The inclusion criteria were non-smoking men and women aged between 35 and 75 years with a waist circumference above the cut-off for high risk of cardio-metabolic disease (193): > 102 cm and > 88 cm for men and women respectively (> 90 cm and > 80 cm, for men and women respectively, with South Asian or East Asian ethnic background). The exclusion criteria included reported kidney or cardiovascular diseases (myocardial infarction, angina, venous thrombosis, stroke, atrial fibrillation, pacemaker), cancer (excluding basal cell carcinoma) in the past 5 years, type 1 diabetes mellitus or type 2 diabetes (fasting plasma glucose ≥ 7 mmol/L), chronic liver disease; previous bariatric surgery or other major surgery (e.g. organ transplantation); significant psychiatric disorder or uncontrolled depression; participation in a weight management drug trial in the previous 3 months; uncontrolled epilepsy; taking medication likely to affect metabolic rate and/or weight (e.g. beta blockers, corticosteroids, diuretics); lactose intolerant; history of substance or alcohol abuse (previous weekly alcohol intake >60 units/men or 50 units/women). Women who were currently pregnant, lactating or planning pregnancy were also excluded.

6.4.5 Recruitment methodology and randomization

Participants were recruited through internal Research Ethics Office recruitment circular e-mail and posters among KCL and through external advertising via two insertions in the

newspaper, one in metro and the other in the evening standard and via advertisement in LighterLife® website and customer's database. The study was also promoted via Facebook, Gumtree and Twitter. Volunteers who responded to advertisements were given more information about the study, completed a recruitment questionnaire via telephone call or e-mail to establish their eligibility for the study and were sent a participant information sheet (**Appendix 9**). Potential participants gave written informed consent and attended a screening visit in the morning upon which anthropometric and blood pressure measurements were taken, and fasting blood sample was collected for assessment of lipid profile, glucose, liver function and haematology to confirm eligibility to take part in the study.

Treatment was allocated using MinimPy 0.3 [9], a computer online programme, by minimization for sex, BMI, ethnicity and waist circumference. The provision of stool samples for further trials was also considered as a factor in the minimization process.

6.4.6 Screening visit

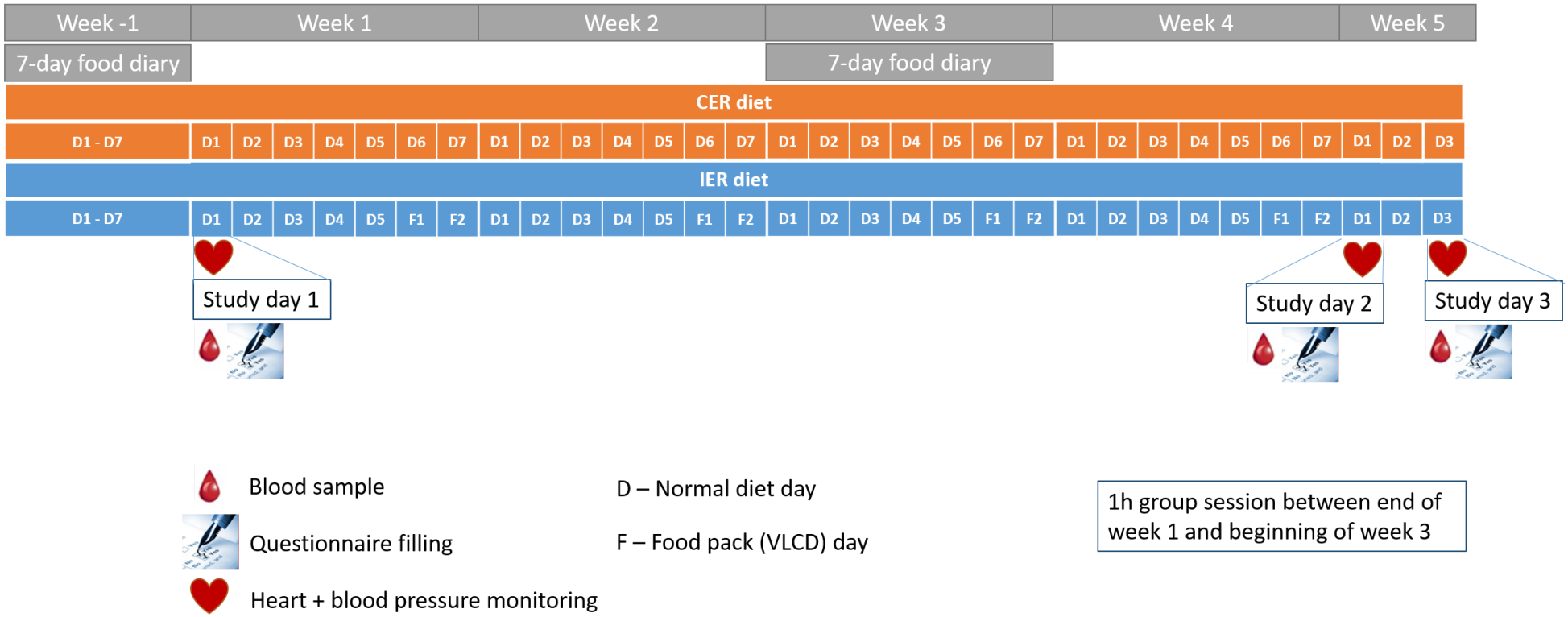
Volunteers were instructed to fast for 12 h before the visit and consume nothing but water until attending the clinic. On arrival, the study protocol was explained in detail and participants were given the opportunity to ask questions before signing the consent form. Once written informed consent was obtained, seated blood pressure was measured in triplicate in the non-dominant arm using an A&D Medical UA-767Plus upper arm automatic blood pressure monitor (A&D Instruments Limited), in accordance with guidelines from the BHS. Height, body weight and percentage body fat and waist circumference were measured using a stadiometer, a Tanita weighing scale (model: BC-418 MA; Tanita UK Ltd) and a tape measure, respectively. A fasting blood sample was taken followed by a light breakfast and the participants were given a 7-day food diary and an International Physical Activity Questionnaire Short Form (IPAQ-SF) to take home and instructed on how to complete it so they could bring it in before the first study appointment. Screening blood samples were sent the same day via courier to Clinical Biochemistry at King's College Hospital, Denmark Hill for analysis.

[9] https://sourceforge.net/projects/minimpy/

6.4.7 Study visits

Outcome measurements were made at baseline and twice at the endpoint (to account for differences at endpoint in the IER group depending on whether measurements were taken immediately after a 2-day SER, or after a non-SER period) after randomization to their respective diets. The CER group were matched to have identical end-point measurements. An overall outline of the study design is given in **figure 25**.

Figure 25 - Overall outline of the study design.



Before the first study visit, participants were instructed to have a standard low fat meal for dinner from a list of ready meals provided. Before all study visits participants were reminded to fast overnight for 12 h and to refrain from alcohol and avoid any strenuous physical activity 24 h before. Also, participants were sent a list of high nitrate foods to avoid eating and were asked to drink Buxton water (low-nitrate water) 24 h before each study visit. The study visits run in the morning, the first lasting around 3h15 and the endpoint visits lasting around 2h30. On arrival participants were reminded of the study day outline and were given the opportunity to ask any questions.

Anthropometric measurements – waist and hip circumferences, height, weight and percentage of body fat - were taken according to the standard operation procedures (refer to **Chapter 2** - Methods, section 2.4). Body fat was estimated using bioelectrical impedance using a Tanita BC-418 MA (Tanita, Arlington Heights, IL, USA). After taking these measurements participants had a 15 min supine rest where the eMotion Faros and ABP monitors were fitted and instructions were give on how to wear them as well as how to complete an activity diary including daily activities, meal and sleep times (**Appendix 10**). Three resting measurements of blood pressure were taken and 30 mins of resting HRV was recorded. While recording the resting HRV measurements, the resting metabolic rate (RMR) was determined using the FitMate^{TM} (Cosmed, Rome, Italy). Also, while resting digital volume pulse measurements were taken. The mental stressor test (the Stroop colour-word test) was performed for 10 mins after the resting period and HRV was recorded during the test. Blood pressure was measured right after the test ended. Both ABP and eMotion Faros Monitors were programmed to record a further 24 h. A Mnemonic Similarity Task (MST) was performed after participants had the opportunity to have a glass of water and unwind from the Stroop test. The MST was included in this study for a collaboration with the Institute of Psychiatry at King's College London (Denmark Hill campus). Data from the MST was analysed by the collaborators and the results will not be presented in this chapter. A blood sample was taken followed by a light healthy breakfast. The blood was processed according to the blood handling protocol (**Appendix 11**). The first study visit (baseline) was then followed by the dietary advice where participants were

revealed the treatment they were allocated to and were given personalised advice and set individual dietary and physical activity goals to follow throughout the intervention.

6.4.8 Estimating total energy expenditure

Total energy expenditure (TEE) was calculated using an adaptation of a previously published template spreadsheet, which identifies the fields needed for data entry by the researcher in the spreadsheet and uses in-build sex-specific algorithms (306). The present study used an adaptation of the template where RMR was measured by indirect calorimetry instead of being estimated through equations built into the template spreadsheet. The following variables were used to calculate TEE: age, weight (kg), RMR (kcal/d) and amount of time spent doing different levels of physical activities (min). The duration of the physical activity levels (walking slowly, walking briskly, moderate and vigorous physical activity) were estimated using an adaptation of the international physical activity questionnaire – short form (IPAQ-SF, **Appendix 12**) which has 7 items and records the activity of four intensity levels: 1) vigorous-intensity activity such as aerobics, 2) moderate-intensity activity such as leisure cycling, 3) walking, and 4) sitting. The participants were further enquired about their type of walking (slow vs. brisk) and of the type of activities practiced for each intensity level to ensure these were correctly labelled. Each activity level was given a metabolic equivalent (METs), which is a value representing the energy cost of physical activities as a multiple of the RMR (307). Based on the compendium of physical activities (308), slow walking was assigned 3.5 METs, walking briskly 4.3 METs, moderate activity 5 METs and vigorous activity 6.5 METs.

The FitMate GS™, a portable desktop indirect calorimeter with a canopy, was used for determining RMR by oxygen consumption. Participants breathed normally inside the canopy, a transparent hood, while in supine position. The canopy was connected to a blower that would provide the air flow inside the hood and the blower pumped in air at different flow rates that could be adjusted depending on participants' weight. The calibration was always done before every measurement and before fitting the hood on the participant.

6.4.9 HRV and ABP measurements

The eMotion Faros devices were used to record HRV in real time during resting and the stress test and on offline mode to record a further 24 h. Skin preparation, monitor fitting and the use of the Cardiscope software has been described elsewhere (**Chapter 2 -** Methods, section 2.1) A&D TM-2430 ABP monitoring devices with appropriate cuff sizes were used to measure resting, immediately after stress test and 24 h SBP and DBP. The ABP monitor was programmed to take measurements every 30 minutes during the day (07:00 to 22:00) and hourly at night (22:00 to 07:00). On completion of the measurements, the eMotion Faros and the ABP devices were collected by courier and returned to KCL. The data were downloaded onto the Cardiscope software and the A&D software from the eMotion Faros and ABP devices, respectively. HRV outcomes were reported for the 24 h measurements, sleep-time and day-time. Sleep times were obtained from the physical activity diary and these entered onto the 24 h recordings in Cardiscope to allow differential analysis of the day/awake and sleep-time. Further analysis was carried on a standardised day-time period of 8 h and sleep-time period of 5 h to remove the influence of variability in recording duration on HRV parameters.

6.4.10 Mental stressor test

The Stroop colour word test was applied as a mental stressor, as it has been reported to experimentally induce stress and cause a shift towards greater sympathetic activity in response to the task (309). The test consisted of two tasks. In the first task, participants were asked to read colour words (red, green and blue) printed in black. Then, in the second task, participants were presented with a sample of colour words printed in coloured fonts, which were incongruent with respect to the colour word itself. They were asked to name the colour font of the printed words and not to read the printed words. Throughout the mental task, the subjects were given instructions by the examiner to work quickly but try not to make any mistakes. The total duration of the mental stress procedure was 10 min.

6.4.11 Blood biochemistry analysis

Blood samples were collected into fluoride oxalate tubes for glucose analysis; SST™ II tubes for full lipid profile (total, LDL and HDL cholesterol and triglycerides), insulin, NEFA, adiponectin, leptin and β-hydroxybutyrate; and EDTA tubes for IL-6 and catecholamines.

Screening blood samples were centrifuged within a maximum of 1 h from collection and sent the same day (within a 5 h window) by a medical courier to the clinical biochemistry laboratory at King's College Hospital. Spare plasma and serum samples were frozen at -40°C. Blood samples from the study visits were centrifuged within a maximum of 30 mins from collection and frozen at -40°C. These samples were sent by a medical courier once the study was completed so all the participants and timepoints were included in the same assay. Plasma glucose and serum lipids were analysed following enzymatic methods using reagents supplied by Bayer Diagnostics Europe Ltd (Bayer House) using an ADVIA 2400 analyser (Siemens Healthcare Diagnostics Ltd, Frimley, Surrey, UK). ELISAs were used to analyse insulin (Siemens Healthcare Diagnostics Ltd), adiponectin and leptin (Quantikine ELISA kits, R&D Systems, Abingdon, UK). β-Hydroxybutyric acid was analysed using an enzymatic assay supplied by Randox Laboratories Ltd (County Antrim, UK) for the ADVIA 2400 analyser. Inflammatory markers interleukin (IL)-6, IL-8, VEGF, IL-1b, IL-1RA, and MCP-1 were analysed using a high-sensitivity cytokine chip array assay (Human cytokine HS X biochip; Randox Laboratories Limited, County Antrim). Plasma free metanephrines (metanephrine (MN) and normetanephrine (NMN), the stable O-methylated metabolites of epinephrine and norepinephrine, respectively) were measured by liquid chromatography-tandem mass spectrometry using TurboFlowTM, an online sample preparation system. Insulin sensitivity was assessed by the revised quantitative insulin sensitivity check index (R-QUICKI: 1/(log glucose (mg/dL) + logInsulin (μU/mL) + logFFA (mmol/L))), and insulin resistance by the homeostatic model assessment (HOMA-IR: Glucose mM x Insulin mU/L / 22.5).

6.4.12 Dietary and physical activity advice and group sessions

Dietary advice was given in order to reduce the weekly energy intake by 3500 kcal, aiming at a weight loss of 2 kg over the 4-week intervention period. Participants allocated to the

CER diet were given dietary advice to reduce their daily energy intake by 500kcal based on a healthy balanced Mediterranean-type diet (**Appendix 13**). Those allocated to the IER diet were instructed to follow a VLCD on 2 consecutive days and to follow a healthy balanced Mediterranean-type diet on the other 5 days, prescribed according to the estimated requirements for an overall weekly deficit of 3500 kcal to match the CER group. Participants were provided with LighterLife® foodpacks (providing ~600 kcal/day and 100% of the RNI for vitamins and minerals) for the 2-d SER days. Each SER day consisted of 4 vegetarian foodpacks and participants were able to choose from a range of different options including milkshakes (chocolate, vanilla, strawberry, banana and caramel), porridge (original or apple and cinnamon), soups (vegetable or tomato and herbs), savoury dishes (curry or spaghetti Bolognese) and bars (chocolate raisin, nut fudge or double chocolate). The nutritional information of these foodpacks can be found in **Appendix 14**.

Participants were given personalised dietary advice based on a 7-day food diary completed before attending the first study visit and set individualised dietary goals as well as one physical activity goal to keep throughout the intervention. The physical activity goal frequency and intensity was adjusted according to the current reported level of activity. For example, for a sedentary individual, physical activity frequency and intensity was gradually increased per week until reaching the UK physical activity guidelines for adults (150 min of moderate intensity activity per week in bouts of 10 minutes or more)[10]; in case of a participant that already followed the guidelines, he/she was requested to maintain the same level throughout the study. Another 7-day food diary was given to complete the week before the last follow-up visit in order to assess compliance to the dietary intervention by the change in energy intake. Mean daily energy intake was estimated using Nutritics dietary analysis software (Nutritics v3.06).

To maximise compliance participants received motivational phone calls and were invited to attend a 1 h group session one to two weeks after the first study visit. These were dynamic sessions including information about portion sizes, weighing portions of different types of foods

[10] https://www.gov.uk/government/publications/uk-physical-activity-guidelines

and comparing to recommendations, an interactive quiz and opportunity to discuss any difficulties or problems following the prescribed diets or share with other participants coping strategies or positive feelings.

6.5 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 22.0 (Statistical Product and Service Solutions; IBM Corp.) on an intention-to-treat basis. Chi-square (χ^2) tests for categorical variables and independent samples *t*-test for continuous variables were used to assess the differences between treatment groups' subject characteristics. Non-normally distributed data were normalised by natural logarithm (LN) before analysis and results were back transformed and shown as geometric means (95% CI).

For the study outcome variables, endpoint (END) was defined as the average of the measurements taken at the two follow-up visits for the CER group and the measurements taken at the follow-up visit after normal eating (at least two consecutive days of normal eating/only MER) for the IER. The SER endpoint is defined as the measurements taken at the follow-up visit after two consecutive days of SER (600 kcal/d).

Baseline results are expressed as means (95% CI) or geometric means. For END analysis, between-group differences of normally distributed data or LN transformed data were tested using univariate ANCOVA, adjusted for baseline values and, for non-anthropometric variables, percentage of weight loss. Day-time and 24 h HRV analysis were also adjusted for physical activity (accelerometry data). The endpoint (END) results are expressed as estimated marginal means (95% CI). Data that could not be normalised by LN transformation were analysed using Mann-Whitney *U* test and significance values are presented unadjusted, with results shown as medians (lower and upper limits of the interquartile range).

To explore the acute effects of SER on HRV parameters, ABP and biochemistry markers, changes between END and SER were assessed in the IER group using paired *t*-test. Data that could not be normalised by LN transformation were analysed using Wilcoxon signed-rank test.

To assess compliance to the dietary intervention, changes in weight, waist circumference and energy intake were assessed using paired *t*-test at baseline and END. To assess compliance with SER, changes in β-hydroxybutyrate in the IER group were assessed using paired *t*-test at END and SER.

6.6 Results

6.6.1 Subjects

Of the 172 participants that responded to the adverts, 56 potential eligible participants attended a screening visit and 45 were eligible and randomised to a treatment group (**Figure 26**). In total, 2 participants from the IER group withdrew from the study (4%) after the baseline visit due to change in availability and the remaining 43 participants completed the study.

Subject characteristics of the 43 participants that completed the study are reported in **table 24**. The CER group was formed of 22 subjects (6 male, 16 female) with a mean age of 56 ± 8 years and the IER group was formed of 21 subjects (6 male, 15 female) with a mean age of 50 ± 12 years. There were no significant differences in mean age, waist circumference or BMI between groups, or distributions of ethnicity and sex between groups. There were also no significant differences in seated resting DBP, SBP and HR between groups or biochemistry measurements (plasma glucose, serum triacylglycerides, serum total-cholesterol, LDL-cholesterol and HDL-cholesterol).

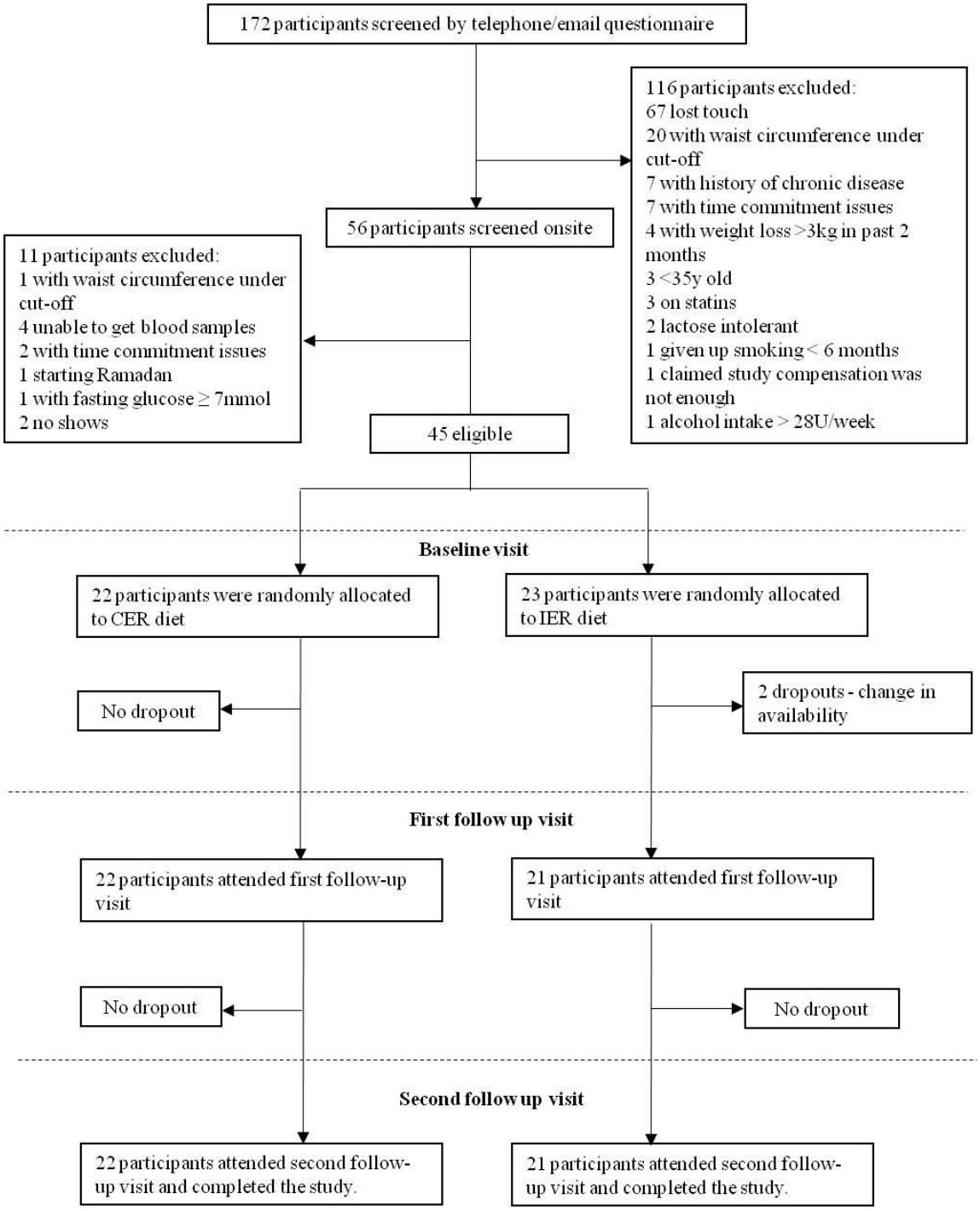


Figure 26 - Study consort diagram.

Table 24 – Subject characteristics at screening visit.

	CER (N=22)		**IER (N=21)**		***p* value** [†]
	n	%	n	%	
Sex					
Male	6	27	6	28	1.000 [§]
Female	16	73	15	72	
Ethnicity					
White	15	68	14	67	0.570 [§]
Black	3	14	1	5	
African/Caribbean					
South Asian	3	14	3	14	
Other	1	5	3	14	
	Mean	SD	Mean	SD	
Age (years)	56	8	50	12	0.097
Waist circumference (cm) [a]					
Male	120 (110, 131)		113 (106, 120)		0.158
Female	108 (100, 116)		105 (99, 110)		0.532
BMI (kg/m^2)	31.1	5.7	31.8	4.5	0.638
% body fat					
Male	31.2	4.2	33.6	6.7	0.476
Female	40.6	5.7	43.1	4.1	0.173
SBP (mmHg)	132	16	127	14	0.325
DBP (mmHg)	88	12	86	8	0.491
HR (bpm)	64.1	7.1	67.0	7.5	0.201
Plasma glucose (mmol/L)	5.4	0.5	5.3	0.4	0.431
Serum TAG (mmol/L) [a]	1.08 (0.88, 1.33)		1.17 (0.96, 1.43)		0.560
Serum total cholesterol (mmol/L)	5.6	0.9	5.4	1.2	0.544
Serum LDL-cholesterol (mmol/L)	3.4	0.7	3.3	1.0	0.719
Serum HDL-cholesterol (mmol/L)					
Male	1.31	0.24	1.35	0.33	0.768
Female	1.80	0.38	1.61	0.42	0.190

BMI, body mass index; DBP, diastolic blood pressure; HDL, high density lipoprotein; HR, heart rate; LDL, low density lipoprotein; SBP, systolic blood pressure; TAG, triacylglycerides

[a] Results expressed as n (%), mean ± SD, except [a] geometric mean (95% CI) and [†] *p* value obtained using independent samples *t* test, except [§] using $\chi 2$ test.

6.6.2 Compliance to dietary intervention

Changes in weight, waist circumference and energy intake were used to assess compliance to the dietary intervention and β-hydroxybutyrate was used to assess compliance with SER in the IER group (**Table 25**). Additional anthropometric measurements that indicate compliance to the dietary intervention are also shown in **Table 25**. Both groups significantly reduced weight, waist circumference, BMI, %BF and energy intake, demonstrating satisfactory compliance to the dietary intervention. The percentage of weight loss was -2.6 ± 1.7 and -2.9 ± 1.6 in the CER and IER groups, respectively. Compliance to SER in the IER group was also achieved as shown by the significant increase in serum β-hydroxybutyrate after the SER endpoint compared to baseline and in agreement with the non-significant difference observed at endpoint in the CER group compared to baseline. However, the CER group presented a significantly higher serum β-hydroxybutyrate at the end of the intervention compared to the IER group (after at least 2 consecutive days of eating normally/MER).

Table 25 – Compliance to dietary intervention, compliance to severe energy restriction and other anthropometry measurements.

	CER (N=22)		Within group: Baseline vs END	IER (N=21)		Within group: Baseline vs END		Within group: END vs SER	Between group treatment effect
Overall compliance	**Baseline**	**END**	***p* value [1]**	**Baseline**	**END**	***p* value [2]**	**2-d SER**	***p* value [2]**	***p* value [3]**
Weight (kg)	89.2 (80.1, 98.2)	86.2 (85.5, 86.9)	<0.001	87.7 (80.2, 95.2)	85.9 (85.2, 86.6)	<0.001	84.7 (77.5, 91.9)	0.003	0.464
Waist circumference (cm) [a]	111 (104, 118)	105 (103, 106)	<0.001	107 (103, 111)	104 (103, 106)	<0.001	103 (98, 108)	0.432	0.535
Energy intake (kcal)	2140 (1833, 2446)	1233 (1068, 1398)	<0.001	2032 (1839, 2224)	1478 (1313, 1643)	<0.001	N/A	N/A	**0.042**
β-hydroxybutyrate (mmol/L) [a]	0.09 (0.07, 0.12)	0.12 (0.09, 0.16)	0.164	0.07 (0.04, 0.11)	0.06 (0.05, 0.09)	0.748	0.20 (0.13, 0.31)	0.001	**0.004**
Other anthropometry measurements									
BMI (kg/m^2)	31.0 (28.4, 33.5)	30.6 (30.4, 30.9)	<0.001	31.9 (29.7, 34.0)	30.5 (30.2, 30.7)	<0.001	30.8 (28.7, 32.9)	0.017	0.340
% BF	37.4 (34.1, 40.6)	36.3 (34.8, 37.8)	0.028	40.0 (37.2, 42.9)	37.5 (35.9, 39.0)	<0.001	38.7 (35.8, 41.5)	0.524	0.357

% BF, percentage of body fat; BMI, body mass index; CER, continuous energy restriction; IER, intermittent energy restriction; 2-d SER, measurements taken following 2-d severe energy restriction; END, measurements at endpoint (not following 2-d SER in case of IER group); BF, body fat; BMI, body mass index; [1] comparison within CER group by paired t-test; [2] comparison within IER group by paired t-test; [3] comparison between groups at END by ANCOVA adjusted for baseline values and, for non-anthropometric outcomes, percentage weight loss. Baseline results expressed as mean (95% CI) and END results expressed as estimated marginal means (95% CI) adjusted for baseline values and, for non-anthropometric outcomes, percentage weight loss, except [a] Geometric means (95% CI).

6.6.3 Resting measurements and mental stress-induced changes in HR and HRV parameters

There were no significant differences between treatments at endpoint on resting HRV measurements, during the mental stressor test, nor in the response to the mental stressor test relative to resting (**Table 26**), except for resting LF power which was significantly higher in the IER group compared to the CER group.

However, the overall effect of both dietary interventions on HRV parameters, independent of the type of treatment followed, produced significant changes in the following resting measurements (mean change [95% CI]): increased IBI (39 [13, 65]) and min IBI (32 [5, 59]) and corresponding decreases in HR (-2.8 [-4.5, -1.0]) and min HR (-1.9 [-3.8, -0.1]).

The following significant changes occurred when the whole cohort was analysed during the mental stressor test at endpoint compared to baseline (mean change in Ln values [95%CI]): increased IBI (mean change [95% CI]; 54 [27, 81]), RMSSD (1.18 [1.03, 1.36]), pNN50 (1.59 [1.14, 2.22]) and HF (1.34 [1.04, 1.72]).

When inspecting relative changes from rest to stress in HR and HRV, assessed by analysing HRV at rest minus Stroop test (10 min recordings), the IER diet at endpoint significantly produced a lower rise in HR in response to stress compared with the CER diet. Although IBI (reciprocal to HR) didn't reach statistical significance between groups at endpoint, only the IER group presented a significantly lower decrease in IBI after treatment. There were no other statistically significant treatment effects noted between groups. When analysing the effect of the weight loss dietary intervention in the whole cohort, the overall intervention significantly attenuated the stress-induced changes on IBI, HR, minHR, LF and HF.

Table 26 –Heart rate variability parameters at rest and during Stroop test measurements at baseline and endpoint.

	CER (N=21)		Within group: Baseline vs END	IER (N=19)		Within group: Baseline vs END	Between group treatment effect	Within all cohort: Baseline vs END
	Baseline	**END**	***p* value [1]**	**Baseline**	**END**	***p* value [2]**	***p* value [3]**	***p* value [4]**
Resting measurements (30 min)								
IBI and HR								
IBI (ms)	958 (921, 996)	974 (942, 1007)	0.099	917 (866, 967)	980 (947, 1013)	**0.018**	0.808	**0.004**
Min IBI (ms)	705 (664, 747)	736 (703, 769)	0.082	692 (650, 734)	718 (684, 752)	0.147	0.457	**0.022**
HR (bpm)	63.1 (60.4, 65.9)	62.3 (60.2, 64.4)	0.101	66.2 (62.7, 69.8)	61.5 (59.3, 63.6)	**0.013**	0.591	**0.003**
Min HR (bpm)	50.7 (47.7, 53.7)	50.3 (47.7, 52.9)	0.444	52.8 (50.2, 55.3)	48.8 (46.1, 51.5)	0.053	0.410	**0.040**
Time-domain HRV parameters								
Ti [a]	13.8 (11.8, 16.1)	14.0 (12.3, 15.9)	0.374	12.8 (11.1, 14.8)	13.7 (12.0, 15.6)	0.805	0.841	0.559
RMSSD (ms) [a]	35.1 (27.5, 44.8)	32.4 (26.6, 39.5)	0.811	29.5 (24.3, 35.9)	38.0 (30.9, 46.6)	0.117	0.272	0.209
pNN50 (%) [a]	8.9 (4.7, 16.7)	7.5 (5.0, 11.3)	0.877	4.9 (2.9, 8.3)	9.5 (6.2, 14.6)	0.056	0.415	0.113
Frequency-domain HRV parameters								
LF (ms^2) [a]	843 (594, 1195)	657 (524, 823)	0.204	598 (413, 868)	956 (759, 1205)	0.073	**0.025**	0.390
HF (ms^2) [a]	390 (227, 670)	356 (255, 498)	0.951	291 (199, 427)	441 (312, 623)	0.104	0.380	0.184
logLF:HF	0.33 (0.15, 0.52)	0.29 (0.18, 0.40)	0.401	0.31 (0.20, 0.43)	0.33 (0.22, 0.44)	0.639	0.598	0.345
Non-linear methods								
SD1:SD2 (Poincaré ratio)	0.32 (0.27, 0.37)	0.29 (0.25, 0.32)	0.374	0.29 (0.25, 0.32)	0.32 (0.29, 0.36)	0.094	0.197	0.763

	CER (N=21)		Within group: Baseline vs END	IER (N=18)		Within group: Baseline vs END	Between group: treatment effect	Within all cohort: Baseline vs END
	Baseline	**END**	***p* value [1]**	**Baseline**	**END**	***p* value [2]**	***p* value [3]**	***p* value [4]**
Stroop test (10 min)								
IBI and HR								
IBI (ms)	883 (842, 925)	926 (888, 965)	**0.012**	861 (809, 913)	922 (882, 962)	**0.011**	0.878	**<0.001**
Min IBI (ms)	708 (670, 746)	745 (708, 783)	**0.049**	713 (679, 747)	714 (675, 752)	0.543	0.883	0.055
HR (bpm)	68.7 (65.3, 72.0)	65.5 (62.4, 68.5)	**0.010**	70.6 (66.6, 74.7)	64.9 (61.7, 68.0	**0.005**	0.779	**<0.001**
Min HR (bpm)	55.9 (52.8, 59.1)	54.1 (51.0, 57.2)	0.100	57.8 (53.8, 61.8)	53.1 (49.9, 56.4)	**0.031**	0.668	**0.006**
Time-domain HRV parameters								
Ti [a]	10.6 (9.0, 12.5)	11.3 (9.8, 13.1)	0.455	10.5 (8.5, 12.8)	11.5 (9.8, 13.4)	0.293	0.910	0.191
RMSSD (ms) [a]	28.0 (22.8, 34.4)	32.1 (26.0, 39.5)	0.094	26.5 (20.4, 34.4)	34.1 (27.4, 42.3)	0.093	0.686	**0.017**
pNN50 (%) [a]	5.5 (3.5, 8.5)	6.7 (4.1, 11.0)	**0.048**	3.4 (1.7, 6.9)	7.7 (4.7, 12.9)	0.074	0.694	**0.007**
Frequency-domain HRV parameters								
LF (ms^2) [a]	639 (479, 852)	830 (644, 1068)	0.080	709 (431, 1165)	767 (590, 997)	0.485	0.667	0.088
HF (ms^2) [a]	218 (135, 352)	314 (214, 459)	**0.050**	213 (124, 366)	286 (193, 425)	0.228	0.738	**0.024**
logLF:HF	0.47 (0.33, 0.61)	0.44 (0.35, 0.53)	0.328	0.52 (0.41, 0.63)	0.44 (0.34, 0.53)	0.159	1.000	0.092
Non-linear methods								
SD1:SD2 (Poincaré ratio)	0.29 (0.23, 0.35)	0.30 (0.25, 0.34)	0.586	0.28 (0.24, 0.32)	0.29 (0.25, 0.33)	0.896	0.800	0.585

	CER (N=21)		Within group: Baseline vs END	IER (N=18)		Within group: Baseline vs END	Between group: treatment effect	Within all cohort: Baseline vs END
	Baseline	**END**	***p* value [1]**	**Baseline**	**END**	***p* value [2]**	***p* value [3]**	***p* value [4]**
Resting – Stroop differences								
IBI and HR								
IBI (ms)	79 (41, 117)	58 (39, 78)	0.145	75 (58, 93)	39 (17, 61)	**0.014**	0.186	**0.006**
Min IBI (ms)	98 (57, 138)	85 (58, 113)	0.578	83 (63, 104)	83 (53, 114)	0.817	0.932	0.563
HR (bpm)	-5.8 (-8.5, -3.1)	-3.8 (-5.0, -2.6)	0.063	-5.8 (-7.3, -4.2)	-1.4 (-2.8, 0.7)	**<0.001**	**0.013**	**<0.001**
Min HR (bpm)	-2.5 (-4.9, -0.2)	-0.8 (-2.4, 0.8)	0.227	-1.7 (-4.2, 0.8)	0.8 (-0.9, 2.6)	0.071	0.171	**0.034**
Time-domain HRV parameters								
Ti	0.5 (-1.4, 2.4)	-0.9 (-2.3, 0.5)	0.333	-1.8 (-3.5, -0.1)	-1.2 (-2.8, 0.4)	0.662	0.760	0.519
RMSSD (ms) [c]	1.05 (0.85, 1.29)	1.01 (0.92, 1.10)	0.910	0.89 (0.78, 1.02)	1.00 (0.90, 1.10)	0.127	0. 858	0.600
pNN50 (%)	9.4 (2.7, 16.1)	3.3 (-1.0, 7.6)	0.076	0.8 (-2.5, 4.2)	1.0 (-3.8, 5.8)	0.707	0.487	0.109
Frequency-domain HRV parameters								
LF (ms^2) [c]	1.05 (0.74, 1.50)	0.74 (0.60, 0.90)	0.074	0.65 (0.48, 0.88)	0.66 (0.52, 0.82)	0.343	0.448	**0.042**
HF (ms^2) [c]	1.90 (1.41, 2.56)	1.30 (1.02, 1.64)	**0.038**	1.39 (0.94, 2.05)	1.25 (0.96, 1.63)	0.436	0.845	**0.038**
logLF:HF	-0.26 (-0.41, -0.11)	-0.23 (-0.34, -0.13)	0.685	-0.33 (-0.45, -0.20)	-0.30 (-0.42, -0.18)	0.842	0.401	0.655
Non-linear methods								
SD1:SD2 (Poincaré ratio)	0.11 (0.04, 0.19)	0.09 (0.03, 0.15)	0.501	0.08 (0.05, 0.12)	0.12 (0.05, 0.18)	0.442	0.604	0.920

CER, continuous energy restriction; IER, intermittent energy restriction; END, measurements at endpoint (not following 2-d SER in case of IER group); IBI, interbeat interval; bpm, beats per minute; HR, heart rate; HRV, heart rate variability; Ti, triangular index; RMSSD, the square root of the mean of the sum of squares of differences between adjacent NN intervals; pNN50, percentage of adjacent NN intervals that differed by more than 50 ms; LF, low frequency power; HF, high frequency power; SD1:SD2, the ratio of the SD of beat-to beat IBI variability (SD1) against the SD of long-term IBI variability (SD2).

[1] comparison within CER group by paired t-test; [2] comparison within IER group by paired t-test; [3] comparison between groups at END by ANCOVA adjusted for baseline values and percentage weight loss; [4] comparison within the whole cohort by paired t-test.

Baseline results expressed as mean (95% CI) and END results expressed as estimated marginal means (95% CI), adjusted for baseline values and percentage weight loss, except [a] Geometric means (95% CI), **[c]** exponents of mean differences in Ln values (the ratio of the geometric mean in resting:that in stroop test, with 95% CI of the geometric mean ratios). Resting – Stroop represents HR/IBI and beat-to-beat HRV during a standardized 10min resting period minus a standardized 10min stress test (Stroop) period, to indicate the difference between rest and stress.

6.6.4 Ambulatory HR and HRV measurements

The ambulatory HR and HRV results are reported for 24 h, day-time (8 h segments) and sleep-time (5 h segments) in **table 27**. There were no significant differences between treatments at endpoint except for SDANN that was significantly higher on the CER group in the sleep-time analysis.

The overall effect of both weight loss dietary interventions combined on HRV parameters, independent of the type of treatment followed, produced significant changes in the following 24h measurements (mean change [95% CI]): increased IBI (20 [0, 40]), SDANN (9.7 [0.5, 19.0]) and VLF (exponent of mean change in Ln values [95% CI]; 1.13 [1.02, 1.25]). HR (reciprocal of IBI) decreased after weight loss, but fell short of significance.

The overall effect of both weight loss dietary interventions on HRV parameters produced significant changes in the following day-time measurements (mean change [95% CI]): increased Ti (3.6 [1.3, 6.0] and VLF (exponent of mean change in Ln values [95% CI]; 1.21 [1.06, 1.39]).

Sleep-time analysis showed a statistically significant increase in pNN50 (exponent of mean change in Ln values [95% CI]; 1.46 [1.06, 2.03]) when analysing the effect of weight loss in the whole cohort. Sleep-time minus day-time differences didn’t produce any significant changes when looking at the cohort altogether.

Table 27 – Ambulatory 24 h, day-time and sleep-time HRV measurements at baseline and endpoint.

	CER (N=22)		**Within group: Baseline vs END**	**IER (N=16)**		**Within group: Baseline vs END**	**Between group: treatment effect**	**Within all cohort: Baseline vs END**
	Baseline	**END**	***p* value [1]**	**Baseline**	**END**	***p* value [2]**	***p* value [3]**	***p* value [4]**
24 h measurements								
Activity, IBI and HR								
Activity level (cpm)	79 (72, 87)	81 (75, 87)	0.923	87 (75, 100)	82 (75, 88)	0.443	0.847	0.631
IBI (ms)	817 (780, 855)	823 (798, 849)	0.122	789 (755, 824)	828 (798, 859)	0.200	0.805	**0.048**
Min IBI (ms)	350 (320, 380)	358 (333, 383)	0.570	366 (331, 402)	381 (352, 410)	0.484	0.230	0.358
HR (bpm)	74.2 (70.6, 77.8)	73.7 (71.4, 76.0)	0.141	76.5 (73.2, 79.8)	73.2 (70.5, 75.8)	0.218	0.756	0.062
Min HR (bpm)	42.5 (39.9, 45.2)	40.4 (38.0, 42.8)	0.187	42.5 (39.7, 45.3)	42.4 (39.6, 45.3)	0.899	0.273	0.214
Time-domain HRV parameters								
Ti	36.5 (32.6, 40.4)	40.2 (36.1, 44.2)	**0.029**	36.5 (31.4, 41.6)	39.0 (34.3, 43.8)	0.492	0.719	0.068
SDNN (ms)	137 (123, 151)	145 (132, 158)	0.069	137 (122, 152)	145 (129, 160)	0.438	0.938	0.123
SDANN (ms)	114 (101, 128)	129 (115, 142)	**0.006**	116 (100, 131)	112 (96, 129)	0.509	0.134	**0.039**
RMSSD (ms)	37.6 (28.1, 47.1)	40.4 (33.5, 47.3)	0.562	41.7 (28.4, 55.0)	43.7 (35.6, 51.8)	0.570	0.537	0.156
pNN50 (%)	12.9 (7.1, 18.7)	14.2 (10.1, 18.3)	0.583	14.1 (6.7, 21.4)	16.4 (11.6, 21.2)	0.443	0.482	0.063
Frequency-domain HRV parameters								
LF (ms^2) [a]	763 (557, 1046)	852 (725, 1000)	0.400	934 (570, 1532)	932 (771, 1125)	0.621	0.472	0.398
HF (ms^2) [a]	336 (216, 523)	430 (326, 567)	0.158	412 (241, 705)	454 (328, 628)	0.451	0.800	0.127
logLF:HF	0.36 (0.24, 0.47)	0.31 (0.22, 0.40)	0.222	0.36 (0.24, 0.48)	0.32 (0.21, 0.42)	0.498	0.908	0.171
VLF (ms^2) [a]	1634 (1376, 1942)	1727 (1520, 1962)	0.170	1556 (1146, 2112)	1910 (1645, 2119)	0.078	0.307	**0.024**
Non-linear methods								
SD1:SD2 (Poincaré ratio)	0.12 (0.10, 0.15) [a]	0.15 (0.12, 0.17)	0.474	0.14 (0.10, 0.18) [a]	0.16 (0.13, 0.19)	0.604	0.551	0.376

	CER (N=22)		Within group: Baseline vs END	IER (N=17)		Within group: Baseline vs END	Between group: treatment effect	Within all cohort: Baseline vs END
	Baseline	**END**	***p* value [1]**	**Baseline**	**END**	***p* value [2]**	***p* value [3]**	***p* value [4]**
Day-time measurements (8h)								
Activity, IBI and HR								
Activity level (cpm)	113 (96, 130)	118 (107, 132)	0.501	114 (98, 130)	117 (105, 130)	0.626	0.916	0.399
IBI (ms)	774 (738, 809)	760 (738, 781)	0.812	733 (697, 769)	774 (750, 799)	0.115	0.368	0.242
Min IBI (ms)	395 (359, 430)	377 (352, 402)	0.238	384 (352, 416)	394 (365, 422)	0.689	0.382	0.609
HR (bpm)	78.4 (74.7, 82.0)	79.9 (77.7, 82.0)	0.763	82.6 (78.5, 86.7)	78.1 (75.6, 80.6)	0.103	0.289	0.223
Min HR (bpm)	49.2 (46.5, 51.8)	48.1 (46.1, 50.1)	0.529	47.4 (43.9, 51.0)	47.3 (45.0, 50.0)	0.577	0.665850	0.401
Time-domain HRV parameters								
Ti	28.8 (25.4, 32.3)	30.9 (27.8, 34.0)	**0.039**	25.5 (21.2, 29.7)	31.2 (27.6, 34.7)	0.044	0.907	**0.003**
SDNN (ms)	112 (100, 125)	113 (103, 123)	0.252	103 (86, 119)	118 (107, 129)	0.100	0.520	0.077
SDANN (ms)	87 (77, 96)	91 (81, 100)	0.127	79 (62, 96)	91 (80, 102)	0.219	0.990	0.058
RMSSD (ms)	33.7 (24.5, 43.0)	35.6 (29.2, 41.9)	0.619	34.8 (23.7, 45.8)	39.0 (31.7, 46.2)	0.395	0.480	0.115
pNN50 (%)	6.3 (3.8, 10.5) [a]	11.4 (7.5, 15.2)	0.063	5.9 (3.0, 11.8) [a]	14.5 (10.1, 18.9)	0.454	0.292	0.089
Frequency-domain HRV parameters								
LF (ms^2) [a]	667 (497, 894)	752 (631, 897)	0.192	761 (452, 1282)	790 (647, 964)	0.548	0.715	0.257
HF (ms^2)	264 (168, 416) [a]	403 (293, 513)	0.117	278 (153, 506) [a]	515 (389, 640)	0.230	0.182	0.052
logLF:HF	0.45 (0.30, 0.59)	0.41 (0.29, 0.52)	0.354	0.47 (0.33, 0.62)	0.36 (0.23, 0.49)	0.240	0.612	0.127
VLF (ms^2) [a]	1483 (1238, 1778)	1476 (1297, 1681)	0.086	1276 (894, 1822)	1746 (1505, 2023)	0.010	0.096	**0.002**
Non-linear methods								
SD1:SD2 (Poincaré ratio) [a]	0.13 (0.11, 0.15)	0.15 (0.13, 0.18)	0.276	0.15 (0.11, 0.19)	0.15 (0.12, 0.18)	0.994	0.760	0.452

	CER (N=19)		Within group: Baseline vs END	IER (N=14)		Within group: Baseline vs END	Between group: treatment effect	Within all cohort: Baseline vs END
	Baseline	**END**	***p* value [1]**	**Baseline**	**END**	***p* value [2]**	***p* value [3]**	***p* value [4]**
Sleep-time measurements (5h)								
IBI and HR								
IBI (ms)	920 (852, 987)	965 (916, 1015)	0.102	949 (884, 1013)	956 (898, 1014)	0.508	0.801	0.125
Min IBI (ms)	578 (513, 643)	549 (514, 583)	0.285	558 (496, 621)	571 (531, 612)	0.423	0.398	0.977
HR (bpm)	66.9 (61.9, 71.9)	63.5 (60.1, 66.9)	0.139	64.0 (59.3, 68.8)	64.0 (60.1, 68.0)	0.649	0.833	0.229
Min HR (bpm)	45.6 (42.3, 49.0)	43.3 (39.9, 46.8)	0.502	43.1 (40.0, 46.3)	44.4 (40.4, 48.4)	0.982	0.695	0.546
Time-domain HRV parameters								
Ti	18.4 (15.3, 22.0)	21.5 (19.4, 23.6)	0.117	21.1 (17.7, 25.0)	17.8 (15.4, 20.3)	0.212	**0.026**	0.927
SDNN (ms)	76 (67, 87) [a]	90 (81, 100)	**0.028**	82 (69, 98) [a]	78 (67, 89)	0.473	0.106	0.393
SDANN (ms)	43.8 (37.0, 50.5)	54.8 (47.6, 62.1)	**0.041**	49.2 (42.7, 55.7)	41.0 (32.6, 49.5)	0.135	**0.017**	0.438
RMSSD (ms)	31.8 (23.0, 43.9)	47.7 (37.6, 57.9)	0.224	42.3 (31.0, 57.7)	48.4 (36.6, 60.2)	0.836	0.934	0.110
pNN50 (%) [a]	8.2 (4.4, 15.0)	14.5 (10.1, 20.7)	**0.015**	11.2 (5.7, 22.1)	12.6 (8.3, 19.1)	0.447	0.606	**0.023**
Frequency-domain HRV parameters								
LF (ms^2) [a]	815 (534, 1244)	911 (708, 1170)	0.373	998 (576, 1729)	951 (709, 1274)	0.980	0.822	0.743
HF (ms^2) [a]	400 (234, 683)	624 (456, 853)	**0.024**	579 (331, 1015)	553 (384, 797)	0.765	0.613	0.091
logLF:HF	0.38 (0.21, 0.54)	0.18 (0.07, 0.29)	**0.029**	0.24 (0.08, 0.40)	0.24 (0.11, 0.36)	0.626	0.502	0.068
VLF (ms^2) [a]	1773 (1407, 2234)	1858 (1566, 2203)	0.463	1715 (1181, 2490)	1799 (1475, 2196)	0.804	0.807	0.495
Non-linear methods								
SD1:SD2 (Poincaré ratio)	0.22 (0.18, 0.28)	0.29 (0.23, 0.34)	0.316	0.26 (0.21, 0.32)	0.31 (0.26, 0.37)	0.165	0.448	0.084

	CER (N=19)		Within group: Baseline vs END	IER (N=14)		Within group: Baseline vs END	Between group: treatment effect	Within all cohort: Baseline vs END
	Baseline	**END**	***p* value [1]**	**Baseline**	**END**	***p* value [2]**	***p* value [3]**	***p* value [4]**
Sleep-time – day-time differences								
IBI and HR								
IBI (ms)	169 (121, 217)	211 (172, 251)	**0.022**	187 (149, 225)	183 (137, 229)	0.862	0.345	0.119
HR (bpm)	-13.7 (-17.4, -10.0)	-16.9 (-19.6, -14.2)	**0.050**	-16.4 (-19.7, -13.0)	-14.5 (-17.7, 11.3)	0.721	0.256	0.332
Time-domain HRV parameters								
Ti	-9.1 (-13.0, -5.3)	-9.1 (-11.9, -6.4)	0.927	-4.7 (-8.8, -0.7)	-11.3 (-14.5, -8.1)	0.053	0.315	0.087
SDNN (ms)	-31.1 (-40.9, -21.2)	-22.3 (-32.3, -12.3)	0.150	-13.5 (-30.2, 3.2)	-34.8 (-46.6, -23.1)	0.070	0.118	0.484
SDANN (ms)	-39.7 (-48.7, -30.7)	-33.5 (-42.9, -24.1)	0.322	-26.0 (-40.4, -11.5)	-46.9 (-58.0, -35.9)	0.036	0.075	0.283
RMSSD (ms)	10.1 (0.6, 19.7)	13.7 (5.5, 21.9)	0.470	13.2 (2.7, 23.7)	7.8 (-0.8, 16.4)	0.662	0.323	0.975
pNN50 (%)	6.8 (0.6, 12.9)	9.9 (4.3, 15.6)	0.212	6.9 (0.6, 12.9)	2.7 (-3.2, 8.7)	0.718	0.085	0.678
Frequency-domain HRV parameters								
LF (ms^2) [c]	1.21 (0.87, 1.69)	1.26 (1.00, 1.59)	0.736	1.32 (0.98, 1.78)	1.29 (0.98, 1.69)	0.997	0.910	0.880
HF (ms^2) [c]	1.75 (1.12, 2.73)	1.99 (1.54, 2.57)	0.626	2.35 (1.44, 3.83)	1.72 (1.28, 2.32)	0.318	0.462	0.626
VLF (ms^2)	396 (-5, 797)	500 (107, 892)	0.757	698 (151, 1244)	143 (-315, -602)	0.146	0.241	0.317
Non-linear methods								
SD1:SD2 (Poincaré ratio)	0.11 (0.08, 0.15)	0.11 (0.08, 0.16)	0.910	0.10 (0.06, 0.14)	0.15 (0.11, 0.19)	0.072	0.407	0.154

CER, continuous energy restriction; IER, intermittent energy restriction; END, measurements at endpoint (not following 2-d SER in case of IER group); IBI, interbeat interval; bpm, beats per minute; cpm, counts per minute; min IBI, minimum IBI; HR, heart rate; HRV, heart rate variability; Ti, triangular index; RMSSD, the square root of the mean of the sum of squares of differences between adjacent NN intervals; pNN50, percentage of adjacent NN intervals that differed by more than 50 ms; LF, low frequency power; HF, high frequency power; SD1:SD2, the ratio of the SD of beat-to beat IBI variability (SD1) against the SD of long-term IBI variability (SD2); SDNN, standard deviation of all NN intervals; ms, milliseconds; SDANN, standard deviation of the averaged NN intervals, calculated from 5 min epochs; VLF, very low frequency power.

[1] comparison within CER group by paired t-test; [2] comparison within IER group by paired t-test; [3] comparison between groups at END by ANCOVA adjusted for baseline values, percentage weight loss and 24 h or day-time activity for 24 h and day-time measurements, respectively, and adjusted for baseline and percentage weight loss for sleep-time measurements; [4] comparison within the whole cohort by paired t-test.

Baseline results expressed as mean (95% CI) and END results expressed as estimated marginal means (95% CI), adjusted for baseline and percentage weight loss, and 24 h or day-time activity for 24 h and day-time measurements, respectively, and adjusted for baseline and percentage weight loss for sleep-time measurements, except [a] geometric means (95%

CI); and [c] Results expressed as exponents of mean differences in Ln values (the ratio of the geometric mean in sleep:that in day, with 95% CI of the geometric mean ratios). Sleep-time – day-time represents HR/IBI and HRV during a standardized 5 h nocturnal sleep period minus a standardized 8 h day-time period, to indicate the difference between night and day.

6.6.5 Acute effects of severe energy restriction on HR and HRV measurements

The acute effects of SER on HR and HRV parameters are shown in **table 28**. Statistically significant acute effects of 2-d SER were observed in the measurements taken during the mental stressor test where significantly lower HR, Ti and HF were observed after 2-d SER compared to non-SER endpoint. IBI (reciprocal of HR) has increased but fell short of significance. However, no differences were found in relative changes from rest to stress after 2-d SER compared to non-SER endpoint, as assessed by resting minus stroop analysis.

No significant differences were found in the IER group between the resting, 24 h and day-time measurements taken after the endpoint visit following 2 consecutive SER days compared to the non-SER endpoint (at least 2 days after the last SER day). The only sleep-time measurement showing a significant change was SDANN which was higher after the SER endpoint compared to endpoint.

Table 28 – Heart rate variability parameters at rest, during Stroop test, and ambulatory measurements over 24 h, day-time and sleep-time: acute response to 2 days SER compared to at least 2 days of normal eating/MER (END) following 4-week intermittent energy restriction dietary intervention.

	END	**2-d SER**	**Δ difference END – 2-d SER**	***p* value[1]**
Resting measurements (N=19)				
IBI and HR				
IBI (ms)	974 (938, 1010)	982 (937, 1028)	-8.2 (-39.6, 23.2)	0.590
Min IBI (ms)	722 (689, 756)	730 (694, 767)	-8.0 (-34.3, 18.3)	0.531
HR (bpm)	61.9 (59.6, 64.2)	61.6 (58.7, 64.5)	0.3 (-1.5, 2.1)	0.723
Min HR (bpm)	49.5 (46.1, 53.0)	49.3 (45.8, 52.9)	0.2 (-1.2, 1.6)	0.748
Time-domain HRV parameters				
Ti [a]	13.1 (11.1, 15.4)	13.8 (11.7, 16.3)	0.95 (0.85, 1.05)	0.297
RMSSD (ms) [a]	36.0 (27.3, 47.3)	38.0 (28.4, 51.0)	0.95 (0.85, 1.05)	0.293
pNN50 (%) [a]	7.7 (4.4, 13.4)	8.1 (4.1, 16.3)	0.94 (0.63, 1.40)	0.753
Frequency-domain HRV parameters				
LF (ms^2) [a]	767 (489, 1202)	815 (488, 1363)	0.94 (0.72, 1.24)	0.641
HF (ms^2) [a]	396 (250, 628)	458 (260, 807)	0.87 (0.70, 1.08)	0.182
logLF:HF	0.29 (0.16, 0.41)	0.25 (0.15, 0.36)	0.04 (-0.05, 0.12)	0.404
Non-linear methods				
SD1:SD2 (Poincaré ratio)	0.31 (0.27, 0.35)	0.31 (0.28, 0.35)	0.00 (-0.03, 0.02)	0.729
Stroop measurements (N=19)				
IBI and HR				
IBI (ms)	921 (872, 969)	945 (902, 988)	-24.5 (-56.1, 7.0)	0.120
Min IBI (ms)	726 (684, 768)	720 (676, 765)	5.2 (-34.2, 44.6)	0.784
HR (bpm)	64.6 (60.7, 68.5)	61.6 (58.8, 64.5)	3.0 (0.2, 5.7)	**0.035**
Min HR (bpm)	53.7 (49.2, 58.2)	53.1 (49.2, 57.0)	0.6 (-2.0, 3.1)	0.644
Time-domain HRV parameters				
Ti [a]	11.4 (9.6, 13.5)	13.5 (11.4, 16.0)	0.84 (0.75,0.95)	**0.009**
RMSSD (ms)[a]	33.0 (24.6, 44.2)	36.9 (27.0, 50.5)	0.89 (0.79, 1.01)	0.068
pNN50 (%)[a]	6.2 (3.2, 11.9)	8.2 (4.2, 15.9)	0.76 (0.48, 1.20)	0.221
Frequency-domain HRV parameters				
LF (ms^2) [a]	807 (467, 1395)	915 (531, 1576)	0.88 (0.67, 1.17)	0.358
HF (ms^2) [a]	283 (155, 515)	399 (212, 749)	0.71 (0.53, 0.94)	**0.020**
logLF:HF	0.46 (0.36, 0.56)	0.36 (0.25, 0.47)	0.10 (-0.02, 0.21)	0.092
Non-linear methods				
SD1:SD2 (Poincaré ratio)	0.29 (0.24, 0.33)	0.30 (0.26, 0.34)	-0.01 (-0.05, 0.03)	0.488
Resting – Stroop measurements (N=18)				
IBI and HR				
IBI (ms) [a]	39 (12, 66)	46 (17, 75)	1.00 (0.95, 1.04)	0.821
Min IBI (ms)	83 (46, 120)	110 (71, 149)	-27.0 (-72.0, 18.0)	0.222
HR (bpm)	-1.3 (-2.6, 0.0)	-0.3 (-0.8, 0.1)	-1.0 (-2.5, 0.5)	0.195
Min HR (bpm)	1.3 (-0.8, 3.4)	-0.9 (-0.4, 7.6)	2.4 (0.0, 4.7)	0.053
Time-domain HRV parameters				
Ti	-1.5 (-3.1, 0.1)	-3.0 (-4.7, -1.3)	1.6 (-0.3, 3.4)	0.089
RMSSD (ms)[a]	-5.0 (-13.0, 3.0)	-3.9 (-8.3, 0.6)	1.00 (0.90, 1.12)	0.981
pNN50 (%)[a]	-0.8 (-4.8, 3.1)	-0.2 (-3.3, 2.8)	1.14 (0.75, 1.73)	0.520
Frequency-domain HRV parameters				
LF (ms^2) [a]	-804 (-1599, -9)	-763 (-1788, 261)	1.00 (0.69, 1.47)	0.984
HF (ms^2) [a]	-152 (-529, 225)	-78 (-227, 71)	1.09 (0.86, 1.37)	0.456
logLF:HF	-0.31 (-0.44, -0.18)	-0.28 (-0.40, -0.15)	-0.03 (-0.22, 0.16)	0.720

	END	2-d SER	Δ difference END – 2-d SER	*p* value[1]
Resting – Stroop measurements (continued)				
Non-linear methods				
SD1:SD2 [a] (Poincaré ratio)	0.12 (0.04, 0.19)	0.12 (0.07, 0.17)	1.00 (0.81, 1.24)	0.997
24 h measurements (N=17)				
Activity, IBI and HR				
Activity level (cpm)	82 (75, 89)	76 (68, 85)	5.6 (-1.9, 13.2)	0.133
IBI (ms)	814 (779, 849)	831 (799, 864)	-16.9 (-47.2, 13.4)	0.255
HR (bpm)	74.2 (71.3, 77.2)	72.5 (69.8, 75.1)	1.7 (-0.8, 4.3)	0.166
Time-domain HRV parameters				
Ti	39.0 (34.0, 44.0)	41.4 (36.5, 46.3)	-2.4 (-9.4, 4.6)	0.476
SDNN (ms)	146 (125, 167)	152 (135, 170)	6.5 (-13.6, 26.6)	0.396
SDANN (ms)	124 (102, 147)	132 (116, 148)	-7.4 (-28.1, 13.2)	0.458
RMSSD (ms)	44.0 (32.2, 55.7)	46.4 (33.5, 59.2)	-2.4 (-12.4, 7.6)	0.617
pNN50 (%)[a]	10.0 (5.5, 18.1)	11.0 (6.2, 19.7)	0.90 (0.47, 1.73)	0.748
Frequency-domain HRV parameters				
LF (ms^2) [a]	1004 (690, 1459)	989 (637, 1537)	1.01 (0.81, 1.26)	0.892
HF (ms^2) [a]	472 (292, 762)	494 (295, 826)	0.96 (0.62, 1.48)	0.830
logLF:HF	0.33 (0.19, 0.46)	0.30 (0.17, 0.44)	0.03 (-0.12, 0.17)	0.703
VLF (ms^2) [a]	1886 (1449, 2454)	1894 (1424, 2519)	1.00 (0.85, 1.17)	0.956
Non-linear methods				
SD1:SD2 (Poincaré ratio)	0.16 (0.11, 0.21)	0.16 (0.12, 0.20)	0.00 (-0.04, 0.04)	0.877
Day-time measurements (8h, N=18)				
Activity, IBI and HR				
Activity level (cpm)	117 (101, 132)	115 (101, 130)	-1.0 (-17.3, 15.3)	0.900
IBI (ms)	762 (724, 800)	757 (720, 794)	6.5 (-21.8, 34.8)	0.636
HR (bpm)	79.4 (75.5, 83.3)	80.0 (76.1, 83.8)	-0.7 (-3.6, 2.3)	0.634
Time-domain HRV parameters				
Ti [a]	28.7 (25.2, 32.7)	28.3 (25.1, 32.0)	1.03 (0.91, 1.16)	0.642
SDNN (ms)	112 (95, 129)	116 (98, 133)	-4.5 (-20.8, 11.7)	0.562
SDANN (ms)	86 (71.3, 100)	89 (75, 103)	-3.5 (-19.0, 12.1)	0.643
RMSSD (ms)	38.0 (26.7, 49.4)	40.2 (27.9, 52.4)	-2.3 (-11.4, 6.8)	0.598
pNN50 (%) [a]	6.6 (3.0, 14.5)	7.9 (3.8, 16.2)	0.83 (0.37, 1.83)	0.622
Frequency-domain HRV parameters				
LF (ms^2) [a]	790 (512, 1220)	755 (472, 1207)	1.03 (0.86, 1.23)	0.718
HF (ms^2) [a]	323 (190, 551)	347 (203, 593)	0.93 (0.60, 1.44)	0.719
logLF:HF	0.39 (0.23, 0.55)	0.34 (0.16, 0.51)	0.05 (-0.10, 0.20)	0.521
VLF (ms^2) [a]	1628 (1200, 2210)	1503 (1053, 2145)	1.07 (0.92, 1.25)	0.342
Non-linear methods				
SD1:SD2 [a] (Poincaré ratio)	0.15 (0.12, 0.20)	0.16 (0.12, 0.21)	0.97 (0.72, 1.31)	0.833
Sleep-time measurements (5h, N=16)				
IBI and HR				
IBI (ms)	958 (897, 1019)	983 (933, 1032)	-24.4 (-86.2, 37.3)	0.413
HR (bpm)	63.5 (59.4, 67.5)	61.6 (58.6, 64.5)	1.9 (-2.3, 6.1)	0.354
Time-domain HRV parameters				
Ti [a]	18.2 (15.8, 20.6)	22.0 (17.5, 26.3)	-3.8 (-7.8, 0.2)	0.059
SDNN (ms) [a]	79.8 (67.9, 93.7)	87.9 (72.8, 106.1)	0.91 (0.79, 1.05)	0.167
SDANN (ms)	42.0 (35.2, 48.8)	53.9 (40.3, 67.4)	-11.9 (-23.8, 0.0)	**0.050**
RMSSD (ms)	53.1 (37.6, 68.5)	55.1 (36.2, 74.1)	-2.0 (-15.5, 11.4)	0.749
pNN50 (%) [a]	14.9 (9.1, 24.2)	13.8 (7.1, 26.7)	1.08 (0.61, 1.91)	0.781

	END	2-d SER	Δ difference END – 2-d SER	*p* value[1]
Sleep-time measurements (continued)				
Frequency-domain HRV parameters				
LF (ms^2) [a]	1191 (774, 1832)	1061 (639, 1761)	1.12 (0.80, 1.58)	0.482
HF (ms^2) [a]	691 (432, 1107)	645 (354, 1177)	1.07 (0.68, 1.68)	0.750
logLF:HF	0.24 (0.09, 0.38)	0.22 (0.07, 0.36)	0.02 (-0.13, 0.17)	0.771
VLF (ms^2) [a]	1974 (1441, 2705)	2153 (1529, 3031)	0.92 (0.69, 1.22)	0.533
Non-linear methods				
SD1:SD2 (Poincaré ratio)	0.33 (0.27, 0.39)	0.31 (0.23, 0.39)	0.02 (-0.05, 0.09)	0.597

IER group only. IBI, interbeat interval; bpm, beats per minute; cpm, counts per minute; min IBI, minimum IBI; HR, heart rate; HRV, heart rate variability; Ti, triangular index; RMSSD, the square root of the mean of the sum of squares of differences between adjacent NN intervals; pNN50, percentage of adjacent NN intervals that differed by more than 50 ms; LF, low frequency power; HF, high frequency power; SD1:SD2, the ratio of the SD of beat-to beat IBI variability (SD1) against the SD of long-term IBI variability (SD2); SDNN, standard deviation of all NN intervals; ms, milliseconds; SDANN, standard deviation of the averaged NN intervals, calculated from 5 min epochs; VLF, very low frequency power.
Results expressed as mean (95% CI), except [a] geometric means (95% CI) [1] comparison within IER group using paired *t*-test, END vs. 2-d SER.

6.6.6 Resting and ambulatory blood pressure

The resting and ambulatory blood pressure results are shown in **table 29**. The ABP results are reported as 24 h, day-time and sleep-time. There were no significant differences in resting SBP or DBP at endpoint between treatment groups although there was an overall decrease in BP following both dietary intervention treatments combined which was statistically significant for resting SBP ($p = 0.032$). Twenty four hour, day-time and sleep-time systolic and diastolic ABP did not differ between groups at endpoint. When analysing the effect of weight loss in the whole cohort, there was a statistically significant decrease in 24 h ($p < 0.001$), day-time ($p = 0.001$) and sleep-time DBP ($p = 0.011$) although no statistical significance was found in SBP.

The IER produced significant acute reductions (after 2-d SER) in resting DBP and borderline significant reduction in resting SBP. No effects were observed of 2-d SER on ABP measurements.

Table 29 – Resting and ambulatory blood pressure measurements.

	CER (N=21)		Within group: Baseline vs END	IER (N=21)		Within group: Baseline vs END		Within group: END vs SER	Between group treatment effect
	Baseline	**END**	***p* value [1]**	**Baseline**	**END**	***p* value [2]**	**2-d SER**	***p* value [2]**	***p* value [3]**
Resting [a]									
SBP	130 (123, 138)	123 (119, 127)	**0.011**	124 (117, 130)	123 (119, 127)	0.477	116 (111, 122)	0.051	0.889
DBP	79 (75, 84)	77 (74, 79)	0.248	77 (72, 82)	77 (74, 79)	0.677	73 (69, 76)	**0.014**	0.916
Ambulatory Blood Pressure									
24 h measurements [b]									
SBP	124 (119, 128)	121 (117, 124)	0.248	121 (116, 126)	119 (116, 123)	0.216	117 (113, 121)	0.257	0.741
DBP	75 (72, 78)	72 (70, 74)	**0.024**	75 (72, 78)	71 (69, 74)	**0.005**	72 (70, 74)	0.435	0.674
Day-time / awake measurements									
SBP	127 (123, 131)	125 (121, 129)	0.521	126 (120, 131)	123 (119, 127)	0.228	120 (1155, 125)	0.344	0.438
DBP	78 (75, 81)	75 (73, 78)	0.069	79 (75, 82)	74 (72, 76)	**0.003**	74 (71, 76)	0.819	0.448
Sleep-time measurements									
SBP	110 (104, 115)	104 (100, 108)	0.089	106 (99, 112)	105 (100, 109)	0.545	105 (99, 110)	0.739	0.928
DBP	65 (62, 69)	61 (58, 63)	**0.026**	61 (58, 65)	60 (57, 63)	0.202	61 (58, 64)	0.664	0.796

DBP, diastolic blood pressure; SBP, systolic blood pressure; 2-d SER, measurements taken following 2-d severe energy restriction; END, measurements at endpoint (not following 2-d SER in case of IER group). [1] comparison within CER group by paired t-test; [2] comparison within IER group by paired t-test; [3] comparison between groups at END by ANCOVA adjusted for baseline values and percentage weight loss. Baseline results expressed as mean (95% CI) and END results expressed as estimated marginal means (95% CI) adjusted for baseline values and percentage weight loss. [a] One missing resting BP measurement in the CER group due to an error that occurred while downloading the data to the computer. [b] 2 missing participants from each group for ABP due to technical problems with the monitor.

6.6.7 Serum and plasma biochemistry markers

Plasma IL-6 and catecholamines (normetanephrine); serum triglycerides, total cholesterol : HDL ratio, leptin and adiponectin; and insulin sensitivity (R-QUICKI) values are presented in **table 30**. There were no significant differences in these markers at endpoint between treatments. The overall effect of weight loss, by analysing the whole cohort together, produced a significant increase in R-QUICKI (mean change [95% CI]: 0.02 [0.01, 0.04]), and significant decreases in triglycerides (exponent of mean change in Ln values [95%CI]; 0.96 [0.93, 0.99]), total cholesterol : HDL ratio (exponent of mean change in Ln values [95%CI]; 0.81 [0.74, 0.88]), leptin (exponent of mean change in Ln values [95%CI]; 0.77 [0.68, 0.86]), adiponectin (mean change [95% CI]: -0.7 [-1.14, -0.18]) and leptin : adiponectin ratio (exponent of mean change [95% CI]: 0.83 [0.74, 0.92]). The IER produced additional significant acute reductions (after 2-d SER) in triglycerides, leptin and the leptin : adiponectin ratio compared to endpoint.

Table 30 – Biochemistry measurements: IL-6, catecholamines, insulin sensitivity, leptin and adiponectin.

	CER (N=22)		Within group: Baseline vs END	IER (N=21)		Within group: Baseline vs END		Within group: END vs SER	Between group treatment effect
	Baseline	**END**	***p* value [1]**	**Baseline [c]**	**END [c]**	***p* value [2]**	**2-d SER[d]**	***p* value [2]**	***p* value [3]**
Plasma IL-6 (ng/L) [b]	0.63 (0.36, 0.88)	0.65 (0.49, 0.96)	0.903 [e]	1.02 (0.60, 1.55)	0.77 (0.63, 1.12)	0.147 [e]	0.88 (0.73, 1.19)	0.354 [e]	0.224 [f]
Plasma Normetanephrine (pmol/L)	489 (380, 597)	563 (428, 699)	0.406	491 (392, 590)	573 (426, 719)	0.255	538 (407, 670)	0.719	0.928
R-QUICKI	0.39 (0.38, 0.41)	0.41 (0.40, 0.43)	**0.013**	0.39 (0.37, 0.42)	0.42 (0.40, 0.44)	**0.006**	0.41 (0.39, 0.44) [a]	0575	0.590
Triglycerides (mmol/L) [a]	1.12 (0.90, 1.39)	0.96 (0.86, 1.06)	**<0.001**	1.38 (1.09, 1.75)	1.04 (0.93, 1.17)	**0.018**	0.98 (0.83, 1.14)	**0.031**	0.270
Total cholesterol: HDL cholesterol ratio [a]	3.52 (3.17, 3.92)	3.44 (3.29, 3.60)	**0.028**	3.36 (3.01, 3.75)	3.51 (3.34, 3.68)	0.262	3.71 (3.40, 4.06)	0.171	0.558
Serum Leptin (µg/L) [a]	18.0 (13.1, 24.7)	15.1 (13.5, 17.0)	**0.009**	23.5 (16.7, 33.0)	16.2 (14.3, 18.3)	**0.001**	14.4 (9.9, 20.9)	**<0.001**	0.448
Serum Adiponectin (mg/L)	8.45 (6.62, 10.34)	7.76 (7.21, 8.32)	0.187	7.91 (5.89, 9.92)	7.31 (6.71, 7.90)	**0.015**	7.41 (5.56, 9.27)	0.613	0.267
leptin : adiponectin ratio [a]	2.40 (1.65, 3.49)	2.23 (1.96, 2.53)	**0.020**	3.53 (2.48, 5.02)	2.56 (2.23, 2.94)	**0.024**	2.34 (1.56, 3.52)	**<0.001**	0.152

CER, continuous energy restriction; IER, intermittent energy restriction; IL, interleukin; 2-d SER, measurements taken following 2-d severe energy restriction; END, measurements at endpoint (not following 2-d SER in case of IER group). [1] comparison within CER group by paired t-test; [2] comparison within IER group by paired t-test; [3] comparison between groups at END by ANCOVA adjusted for baseline values and percentage weight loss. Baseline results expressed as mean (95% CI) and END results expressed as estimated marginal means (95% CI) adjusted for baseline values and percentage weight loss, except [a] geometric mean (95% CI) and [b] median (lower and upper IQR); [c] 2 missing samples in IER group, n=19; [d] 1 missing value, IER n=20; [e] *p* value obtained using related samples Wilcoxon Signed Rank Test; [f] *p* value obtained by Mann-Whitney *U* test.

6.7 Discussion

This is the first RCT assessing the effects of intermittent fasting on HRV that recruited healthy centrally obese men and women based on waist circumference ethnicity specific cut-offs for high risk of cardio-metabolic disease. The IER intervention group was compared with an isoenergetic CER control group in a parallel design. Both treatments achieved comparable weight loss and waist circumference reductions which was the aim of the trial so that changes in the outcome variables could be attributed to the type of energy restriction diet followed as opposed to weight loss alone.

In otherwise healthy individuals, obesity has been shown to decrease HRV (310) and that losing weight can improve HRV in obese and normal weight individuals as well as diseased states (hypertension, type 2 diabetes; please refer to Chapter 1, section 1.5.1.2). However, preliminary data from the LighterHeart Study in our group showed that favourable changes in ANS function occur rapidly following onset of SER (within 1 week) and these could be through a weight-loss independent mechanism. Therefore, this study tested whether an IER approach for weight loss would confer any additional cardiometabolic benefits compared to a CER approach in centrally obese individuals, independent of weight loss. It was therefore hypothesised that losing weight via an IER approach would have superior benefits for the cardiac autonomic system (assessed by HRV), compared to a CER approach. However, this study showed no superior effects of following an IER diet compared to a CER diet on HRV at rest, during a mental stress inducing test, or in ambulatory measurements. Any observed improvements in HRV were due to overall weight loss. Analysis of the acute effects of SER in the IER group showed lower stress-induced changes in HRV after two consecutive days of SER.

6.7.1 Resting and stress-induced changes in heart rate variability measurements

In a controlled setting, resting measurements were recorded followed by a mental stressor test to record stress-induced changes in HRV. These changes recorded in a controlled setting explored the effects of a mild stressor on autonomic changes with minimal potential confounders as opposed to a free-living situation. From the measurements taken under

controlled conditions, there were no significant differences at endpoint between treatments, except for resting LF power which was significantly higher in the IER group compared to the CER group; and HR increase in response to stress was significantly attenuated in the IER group compared to the CER group. The studies assessing HRV in intermittent fasting are limited and come from rodent experiments. Mager et al measured 24 h HRV in rats maintained on an intermittent fasting diet (every other day feeding) or energy restricted diet (40% energy reduction) and showed an increase in LF and HF power of HRV (with a stable LF:HF ratio) that was comparable between the two diets, and maximal effects achieved after 4 to 5 weeks (296). The greater LF power in the IER group was not accompanied by a significant increase in HF but preserved the LF:HF ratio. Although there were no differences between groups in IBI (reciprocal to HR) stress-induced decrease, within the IER group the stress induced decrease in IBI was significantly reduced. This effect of intermittent fasting on cardiovascular adaptation to stress has been previously described in rats subjected to stress (immobilization and cold-water swim) where the rats on intermittent fasting had a lower stress-induced increase in HR compared to a control *ad libitum* diet (311). Acutely, SER further reduced the stress-induced increase in HR and attenuated the stress-induced decrease in overall HRV, as shown by a significantly lower stress-induced decrease in Ti after SER compared to non-SER endpoint, and by preserving better the vagal tone, shown by a lower stress-induced decrease in HF. A potential mechanistic explanation for this finding in the IER group could involve the enhanced activity of brainstem cholinergic cardiac vagal neurons - which increase parasympathetic tone - by the brain-derived neurotrophic factor (BDNF) (312). BDNF expression and signalling have been shown to increase in response to physical activity and intermittent fasting (313) and Wan et al were able to provide evidence for the first time in mice of its role in the regulation of HR by enhancing the activity of cardiovagal neurons in the nucleus ambiguus of the brainstem, which in turn send their axons through the vagus nerve to the heart releasing acetylcholine that has a HR lowering effect (314).

The overall weight loss effect, independent of the type of diet followed, resulted in statistically significant increases in resting IBI and minIBI, and decreases in HR and min HR.

The IBI and HR changes were consistently greater after following the IER diet compared to the CER diet. The overall weight loss effect also resulted in attenuated stress-induced changes during the stressor test in HR/IBI and beat-to-beat HRV (pNN50, RMSSD and HF - representing parasympathetic tone). Further analysis showed that the IBI/HR stress induced changes were more attenuated in the IER group whereas the stress-induced beat-to-beat HRV changes were more attenuated in the CER group. These observed changes in the response to the stress test after the intervention could be the effect of weight loss or could also be habituation to the mental stress test. The greater perseverance of the parasympathetic tone observed in response to the mental stress test confers a more flexible autonomic regulation which in turn represents a better cardiovascular resilience and adaptation to mild stress following weight loss.

6.7.2 Ambulatory heart rate variability measurements

From the ambulatory measurements, there were no significant differences between treatment groups at endpoint, except for nocturnal SDANN, a measure of longer-phase variability, which was significantly lower in the IER group compared to the CER group. However, this finding may be a multiple testing type I error as VLF, the other HRV parameter reflecting longer-phase variability, did not show any significant differences between groups.

The overall effects of weight loss show statistically significant increase in 24 h IBI, and a trend towards a decrease in HR, that are comparable to the results observed after a 12-week continuous energy restriction weight-loss programme in overweight women, that were otherwise healthy (103). This study also observed a significant increase in SDNN after weight loss, which was not observed in our findings, except for a trend towards an increase in SDNN in the day-time. However, our findings showed a statistically significant increase in 24 h and day-time SDANN (borderline significance in day-time) and VLF after weight loss, which represent the longer-phase variability arising from hormonal changes, including the renin-angiotensin-aldosterone system and thermoregulation. An increase in 18 h VLF power was observed in a study with severely obese normotensive subjects after a 3 week dietary and high intensity aerobic physical activity intervention, along improvements in vagally-mediated parameters (RMSSD, pNN50 and HF) (100). Another small study (n = 8) in severely obese subjects (≥ 40

kg/m^2) showed an increase in 24 h and day-time IBI, SDANN and VLF after 3 months on a weight loss program (varying between 1000 – 1500 kcal / day provided with foodpacks – percentage of energy restriction was not mentioned) (99). In our study, a statistically significant increase in Ti after weight loss was seen in the day-time, and a trend of an increase in 24 h period. The only study showing an increase in Ti after weight loss comes from a study in women with bariatric surgery-induced weight loss, over 24 h measurements (127).

6.7.3 Resting and ambulatory blood pressure

No differences at endpoint were seen between groups in resting or ambulatory BP. The only other published RCT in humans that compared directly the effects of a CER and IER diets in metabolic disease risk with the same protocol used in this study (2 consecutive days of a VLCD per week) was done in healthy overweight women with an increased risk of breast cancer (299). This study measured resting BP and found small reductions in systolic and diastolic BP that were not different between groups at endpoint which is consistent with the resting BP results in this study. A study comparing alternate-day fasting (25% energy needs on fast days and 125% energy needs on alternating "feast days") with CER (25% daily energy restriction) also reported no differences in resting HR, systolic and diastolic BP between treatment groups after 6 months of intervention (315). An 8-week intermittent fasting study with 30% energy restriction (6 days energy restricted + 1 day fasting - 120 kcal juice powder for 24 h) where one group (IFCR-L) had liquid meal replacements for breakfast and lunch and a food based dinner on energy restricted days and the other group (IFCR-F) had all three meals food based on energy restricted days. Both groups had a non-significant reduction in resting SBP but only the IFCR-L group additionally presented a non-significant reduction in resting DBP which is in line with the results obtained in both groups in this study. The IFCR-L group achieved a greater energy restriction that was reflected in a significantly higher weight loss which is more comparable to the energy restriction achieved by this study's cohort (316).

6.7.4 Biochemistry measurements

In the present study serum leptin significantly decreased in both groups after the dietary intervention and had further significant reductions in the IER group when measured after the

two consecutive days of SER. The significant decrease in leptin concentrations observed in both groups is likely to be related to the comparable reduction in fat mass by both groups (317). Klempel et al also measured adiponectin and leptin which showed both significant decreases within each group (316). There is strong evidence showing an increase in leptin, with the activation of the brain melanocortin system, linking obesity with overactivation of sympathetic renal activity and elevated blood pressure (318). A decrease in leptin may be one of the mechanisms responsible for the blood pressure lowering-effects of weight lost in central obesity and the fact that the IER regime presented further significant reductions in leptin after 2-d SER could be associated with the greater reductions seen in 24 h and day-time systolic and diastolic BP following 2-d SER. Although both treatments resulted in decreases in adiponectin, only the IER produced significant decreases within group. It's been shown that adiponectin acutely decreases in response to the first 8-12 weeks of energy restriction and tends to increase once a 10% of weight loss has been achieved (319).

The significant increase in ketone bodies after two consecutive days of SER, measured by an increase in serum β-hydroxybutyrate, indicates a switch in metabolic mode from glycogenolysis to beta-oxidation of fatty acids. β-hydroxybutyrate has been shown to inhibit SNS activity at the level of sympathetic ganglion in mice as an antagonist for GPR41 and may also have a direct inhibitory effect on the heart rate (132). This could be a potential mechanism for the beneficial acute effects of fasting in preserving HR and HRV under stress conditions.

6.7.5 Other considerations

In rodents, the cardiovascular system response to IER (reduced resting HR and BP) is reversed within 1 to 2 weeks of going back to an *ad libitum* diet indicating that the cardioprotective benefits are not sustained in the long-term once the IER is discontinued (296). Studies on weight-maintenance in intermittent fasting are scarce and the few that have been performed have shown the reductions in weight to be maintained in both groups, although this was assessed one month after the termination of the intervention (299), or showed better weight-maintenance for the alternate-day fasting group 6 months after the intervention (320). Whether

the effects of intermittent fasting are sustained in the weight-maintenance phase should be addressed in the future.

6.7.6 Strengths and limitations

This study has a few strengths, namely the fact that ambulatory measurements were taken for blood pressure, HR and HRV so there is no influence of the white-coat hypertension (321), and the IER diet was directly compared to the CER diet. This study also has a few limitations, namely the short duration of the dietary intervention (4 weeks), although this was a mechanistic study based on a 1-week acute pilot study (the LighterHeart study) that didn't provide a justification for a longer intervention. This study lacked a control group where participants weight was stable, hence the overall weight loss effects presented needs further confirmation by another study in order to rule out a habituation effect of wearing the monitors. The acute effect of ambulatory measurements is actually a carry-over effect as the ambulatory measurements started while participants were fasted but recorded further 24 h when the participants would have started the non-SER eating days. Physical activity was measured for 24 h while wearing the heart monitor (which had an incorporated accelerometer where physical activity data was obtained from) and the ABP monitor which may not represent the overall physical activity levels taken throughout the intervention. Another limitation is the fact that HRV and ABP measurements were done simultaneously and due to some volunteers having their sleep interrupted while the blood pressure monitor was taking a reading, this could have interfered with the HRV measurements.

In summary, this study showed no superior effects of weight loss achieved through an IER diet compared to a CER diet on HRV at rest, during a mental stress inducing test, or in ambulatory measurements. The improvements in HRV observed were due to overall weight loss effect and there was no evidence of an independent effect of energy restriction pattern. Analysis of the acute effects of SER in the IER group showed lower stress-induced changes in HRV after two consecutive days of SER. An IER approach to weight loss appears to be an alternative to a CER approaches with comparable weight loss and body composition changes. IER could

potentially be used as part of a healthy lifestyle for weight-maintenance if this regime suits best certain individuals' lifestyle. Further RCTs are needed with longer durations and assessment of cardio-metabolic outcomes in a weight-maintenance phase to assess whether the changes observed could potentially be sustained in the long term.

Chapter 7 Discussion

This thesis broadly explored diet as a determinant of cardiac autonomic function by investigating relationships between HRV and aspects of diet / nutritional status that could influence sympathetic and parasympathetic activity in healthy and diseased states.

Cardiac autonomic function is a neglected aspect of the pathophysiology of CVD in the field of nutritional sciences, and there is relatively little published literature on how whole diets or dietary components may influence this important CVD risk factor, compared to other risk factors such as blood lipids, insulin resistance and blood pressure. The overall aim of the thesis was to explore the associations and impacts of some of the main dietary candidates that may influence HRV: LC *n*-3 PUFA and energy intake restriction.

7.1 Method development for assessing HRV in nutritional sciences research

In nutritional sciences research, the use of low-burden HRV measuring devices is advantageous as participants are required to wear them in the free-living state. In contrast to the widely used Holter monitors, the Actiheart and eFaros devices are small, chest worn devices with incorporated accelerometers that, when used in free-living populations, do not interfere with subjects' daily activities, hence not altering their usual routine. Throughout the doctoral work, two different methods for measuring HRV were used. The first method used Actiheart devices which only stored IBI data and required a substantial amount of manual editing of data to remove artefacts and quite a few recordings had to be excluded due to too much unusable data either derived from loss of signal or too much noise. This method was further improved by testing new ECG pads in the reproducibility study and using those that produced an overall better signal to noise ratio in the Om3ga and FISHH studies, although the amount of editing was still substantial and very subjective. The second method used for assessing HRV was greatly improved due to the acquisition of a new recording device (eFaros) that had just become available for the last study, the Met-IER. This method substantially reduced the subjectivity of the manual component of HRV data processing through improvement in software analysis,

while still allowing for visual inspection of the raw ECG data and manual edits if/where needed. This method was suitable for measurement of acute changes, for example during a mental stress test, as well as ambulatory 24 h monitoring, it demonstrated detection of increases in mean IBI and longer-phase HRV in response to weight loss across the study population, and could be appropriate for use in further dietary intervention studies. Limitations to the application of this approach for assessing cardiac autonomic function in future dietary intervention studies include the large number of parameters generated, rendering the interpretation of such sizable dataset complex and vulnerable to type I errors. Further work is required to identify the most clinically meaningful and sensitive parameters for use in detecting the subtle physiological effects expected from dietary interventions.

7.2 LC *n*-3 PUFA and HRV

Initially, HRV data collected from the MARINA trial, a RCT assessing the effect of fish oil supplementation equivalent to eating 0, 1, 2 or 4 portions of fish per week, was analysed for the day-time period, showing that there may be a treatment effect of an increase in longer-phase HRV similar to that previously shown and published for the sleep-time period (186). However, the population studied appeared to be replete in LC *n*-3 PUFA from the start of intervention, and therefore unlikely to respond in the same way as populations that have low LC *n*-3 PUFA status. This led on to the first observational study (the Om3ga study) comparing HRV in vegans with age/sex/BMI-matched omnivore controls, representing populations with low and high tissue LC *n*-3 PUFA status, respectively. It was hypothesised that vegans would display lower mean HRV, however, the observed differences between groups were more complex, with vegans presenting higher 24 h overall HRV due to greater sleep-day differences compared to omnivores, and lower beat-to-beat HRV in the day-time. Vegans also presented lower availability of LC *n*-3 PUFA lipid mediators that may influence anti-inflammatory capacity, especially in populations with underlying chronic low-grade inflammation that present low tissue LC *n*-3 PUFA status. This led to a feasibility study to characterise the variability of LC *n*-3 PUFA status in a sample of stage 5 CKD patients commencing haemodialysis, which is a population with a high burden of inflammation and CVD compared to the general population, with the aim of establishing a

potential relationship with HRV. Haemodialysis patients in this study had particularly low proportions relative to total fatty acid content and a narrow degree of variability of erythrocyte membrane EPA + DHA, which precluded any meaningful findings in the relationship between tissue LC *n*-3 PUFA status and HRV. Nevertheless, analysis of the plasma LC *n*-3 PUFA, a biomarker of short-term LC *n*-3 PUFA intake, showed that EPA (but not DPA or DHA) proportions in plasma, was associated with better overall and longer-phase autonomic regulation. However, EPA was associated with reduced beat-to-beat regulation, which presented the same trend for DPA, which suggests that a compromised conversion of DPA to DHA might be a potential mechanism whereby these patients have depressed parasympathetic tone, either due to the reduced availability and incorporation of DHA into the cardiomyocytes membranes and/or due to a reduction in DHA-derived lipid mediators involved in neuroprotective processes in this patient population. The Om3ga study showed that vegans - as a model of a population with low LC *n*-3 PUFA tissue levels - had reduced availability of precursor markers for pro-resolving lipid mediators. As a healthy population, this might not necessarily have a significant impact on cardiovascular events or SCD but in populations at higher risk of cardiovascular disease and SCD such as CKD, having low LC *n*-3 PUFA tissue levels as it was observed in the FISHH study (median O3I 3.26%) may have severe repercussions. In addition, the plasma DHA in this haemodialysis population was lower than the one found in the omnivore subjects from the Om3ga study, although the estimated DHA dietary intake was comparable, which indicates a compromised metabolism and/or incorporation of the LC *n*-3 PUFA in tissue, which is likely to be at the level of interconversion from DPA to DHA, suggested by the FISHH study results.

The pathophysiology of CVD observed in patients with stage 5 CKD is characterised by atherosclerosis caused by excess LDL-cholesterol and vascular inflammation (322). The fact that the FISHH study population presented very low erythrocyte and plasma proportions of DHA and EPA, and potential compromised interconversion of DPA to DHA, indicates reduced availability of lipid mediator precursors involved in the resolution of inflammation. Reduced availability of SPM precursors would be likely to potentiate vascular inflammation and is implicated as one of the potential mechanisms in the accelerated atherosclerosis observed in this

population (323). A RCT in stage 3 and 4 CKD patients showed that *n*-3 PUFA supplementation reduced plasma 20-HETE (324), an AA-derived lipid mediator previously shown to enhance vascular inflammation in animal models of diabetes and ischaemia/reperfusion (325). The same trial has also shown that supplementation with *n*-3 PUFA significantly increased RvD1, and precursors of the E- and D-series resolvins 18-HEPE and 17-HDHA, respectively, which are involved in actively promoting the resolution of inflammation (326). Therefore, it could be argued that a fish oil supplementation study to increase EPA and DHA tissue levels in haemodialysis patients is clinically justified in order to reduce risk of cardiac mortality/events. The cardiovascular effects of LC *n*-3 PUFAs in haemodialysis patients have not been extensively explored, as discussed in the introduction of Chapter 6 (section 6.1), and studies relating LC *n*-3 PUFAs tissue status to HRV are scarce (106,111), which warrants further studies in CKD patients to assess the relationship between HRV and LC *n*-3 PUFA tissue status using other potential biomarkers of intake, such as mononuclear cells and adipose tissue. Adipose tissue fatty acid composition is reflective of long term dietary intake with an estimated average $t_{1/2}$ of 6-9 months of intake, and has been considered the gold standard measurement for dietary fatty acid status (221). Exploring the use of adipose tissue for *n*-3 PUFA status in patients that recently started haemodialysis may inform of their longer-term status and using this method to measure LC *n*-3 PUFA status wouldn't be affected by the uraemic conditions. Mononuclear cells have more recently been suggested as the most suitable biomarker for long-term oily fish intake within the range likely to be consumed by the UK population (1-4 portions/week) (291). This biomarker has not been used previously in a haemodialysis cohort to assess LC *n*-3 PUFA status and may be explored as an alternative to erythrocyte membrane, as the analysis of the latter biomarker presented challenges in this population.

Venous blood plasma concentrations of lipid mediators in whole fasting plasma are likely to be an insensitive marker of capacity for autacoid release and activity in specific sites of inflamed tissue. Nevertheless, higher circulating plasma concentrations of SPM precursor markers may indicate ease of bioavailability for conversion to SPM when needed, which

presents clear functional implications for populations with low tissue EPA and DHA stores (227). It is not known whether individuals with low *n*-3 PUFA status have increased rates of EPA-/DHA-derived mediators turnover as an adaptive mechanism to avoid compromising SPM availability. If this were the case, then it would be expected that having low pools of SPM precursors would have no functional consequences in vegans where the inflammatory burden is generally low, but could carry consequences in haemodialysis patients where the underlying chronic inflammation might not resolve due to not having enough tissue EPA+DHA that could be metabolised to anti-inflammatory lipid mediators. Future research in this area should address whether populations with low *n*-3 PUFA status are more at risk of having a pro-inflammatory profile, independent of underlying diseased states. In order to provide convincing evidence for the potential efficacy of supplementation with EPA+DHA in increasing HRV, RCTs are needed in populations with low tissue LC *n*-3 PUFA status rather than populations with above average proportions of EPA+DHA in erythrocyte members (>5%).

7.3 Energy restriction/fasting and HRV

Obesity is characterised by a chronic low-grade inflammation and is thought to increase the risk of SCD independently from other cardiovascular risk factors (327). Furthermore, there is a small amount of evidence from animal studies that a ketone body derivative, β–hydroxybutyrate, can inhibit sympathetic activity, which formed the rationale for the Met-IER study, a RCT in centrally obese, and otherwise healthy subjects, to compare the effects of intermittent and continuous energy restriction diets on HRV, independent of weight loss. It was hypothesised that beat-to-beat HRV would increase to a greater extent following IER compared to CER despite equivalent weight loss, and it was theorised that this might be linked to raised ketone production. Contrary to the hypothesis, the results showed no superior effects of weight loss via an IER diet compared to a CER diet on HRV, but indicated an overall effect of weight loss in improving HRV, insulin sensitivity, BP and leptin, suggesting that weight loss itself may have a much greater effect on HRV and other markers of cardio-metabolic risk than any independent effect of energy restriction, or patterns of energy restriction. The possibility remains that the increase in ketone production/severity of the energy restriction was not enough

to trigger the postulated pathways that might inhibit sympathetic firing. This could be further investigated by comparing ketogenic (low carbohydrate) diets with normal moderate carbohydrate diets with the aim of maintain energy balance in a centrally obese study population to explore whether this had any effect on HRV parameters. Future studies will also need a longer duration to assess any differential effects of losing weight through CER or IER on HRV and cardiometabolic risk factors.

7.4 Inflammation as a mediator of dietary impact on autonomic nervous system function

Chronic low-grade inflammation is a unifying component of the diseased states explored in this doctoral research (CKD and central obesity) and could be a key mediating mechanism for the postulated impact of diet on the ANS, and consequent risk of SCD.

A limitation of nutrition studies assessing the impact of diet on inflammation is the fact that the inflammatory markers are often measured in fasting blood samples, which may not necessarily reflect inflammation in tissue compartments, nor the inflammatory response to challenges (328). There are only a few studies in humans addressing the influence of diet on lipid mediators, and the Om3ga study was the first to publish results on LC *n*-3 PUFA-derived lipid mediators in vegans. Although the analysis was performed in fasting plasma, these indicated a significant difference in lipid mediator patterns between vegans and omnivores, being the first step towards the understanding of the impact of a low *n*-3 PUFA tissue status in the production of precursors of SPM (as SPMs were not detected) that have been shown to be actively involved in the resolution of inflammation. This study provides directions for future studies, such as postprandial measurement of lipid mediators after a high-fat meal challenge in vegans compared to omnivores. Inducing obesity in mice was shown to significantly reduce 17-HDHA and dietary treatment with *n*-3 PUFA increased 17-HDHA and reduced obesity-associated adipose tissue inflammation (329). A RCT assessing the effect of LC *n*-3 PUFA supplementation in severely obese nondiabetic patients showed increased production of anti-inflammatory eicosanoids (RvE1, 17-HDHA, PD1 and RvD1) in visceral adipose tissue, and decreases in IL-6 in the LC *n*-3 PUFA supplemented group compared to the control group

(330). In addition, RvE1 and RvD1 was only detected in the adipose tissue from the LC *n*-3 PUFA supplemented group, which might explain why resolvins were not detected in the Om3ga study omnivores. Since the production of SPM precursors has been associated with LC *n*-3 PUFA tissue status, shown by the results of the Om3ga study, LC *n*-3 PUFA supplementation may lower inflammatory burden and thus have a beneficial impact on the ANS function. The modest weight loss achieved over 4 weeks in the Met-IER study did not significantly reduce plasma IL-6. However, the effects of weight loss in SPMs production have not been fully investigated, and should be considered in future studies together with measurements of a broader range of inflammatory markers.

7.5 Future work directions

There is the potential of LC *n*-3 PUFAs and weight loss (independent of the type of diet) to improve cardiac autonomic function although further research is needed to 1) clarify potential benefits of supplementing populations with low LC *n*-3 PUFA status, such as populations deemed to be healthy (vegans) or those with a disease that carries a greater inflammatory burden compared to the general population, such as stage 5 CKD. Future work should include a 4-arm RCT to test the effects of LC *n*-3 PUFA supplementation (algal source) on HRV and lipid mediators in vegans (intervention and control group) and in omnivores (intervention and control groups) where low O3I (<5%) would be an inclusion criteria; future work should also directly compare the effects of fish oil vs. drugs used in primary prevention of CVD (such as statins and β-blockers) vs. combined treatment on HRV and inflammatory markers in participants screened for low O3I (<5%). 2) explore alternative biomarkers for tissue LC *n*-3 PUFA status in haemodialysis patients. Future work should assess the potential of other biomarkers of *n*-3 PUFA intake (such as adipose tissue and mononuclear cells) and assess their relationship with HRV, as well as assess the effects of DHA supplementation on HRV in haemodialysis patients as the FISHH study has suggested a compromised metabolism in the DHA synthesis pathway; 3) further explore the effects of intermittent fasting in RCTs with longer duration. Future work should assess the effect of IER on HRV using a RCT (with a minimum of 6 months, ideally 1 year) including a CER group and a control group where weight

loss does not occur. There should be a follow-up after the first month so that the effects of short-term intermittent fasting can be compared with the longer-term effects. In addition, the effects of intermittent fasting should be further explored in other population groups with central obesity that also have additional comorbidities (such as renal disease and hypertension).

This doctoral research consisted mostly of pilot investigations, and has added a contribution towards the understanding of how diet may play a role in modulating ANS through modulating inflammation, with an eventual role in preventing CVD and SCD. The findings provide preliminary data that might benefit the dietary supplements/nutraceuticals industry with regards to the importance of increasing *n*-3 tissue status in non-fish-consuming populations, and provides valuable information for the medical community, particularly healthcare professionals who specialise in renal medicine. It also provides initial evidence that can be used by dietitians, as part of a growing body of literature, regarding the cardiovascular health benefits of different dietary strategies for weight loss. Finally, this work has opened future directions for research into the potential role of dietary interventions in preserving or improving cardiac autonomic function, including testing the effects of energy restriction alone or in combination with LC *n*-3 PUFA supplementation in SPMs production, inflammatory markers and HRV to assess a potential causal association between these components of diet with ANS activity.

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Appendices

Appendix 1 – Recommendations for EPA and DHA intake in adults.

Organisation	Target population	Recommendation
World Health Organisation (WHO) / Food and Agriculture Organization (FAO) (331)	**General population**	**LC n-3 PUFA: 0.5-2% daily energy intake** **EPA+DHA: 250 mg/day**
National Heart Foundation of Australia (332)	**General population (primary prevention of CHD)**	**Fish intake: 2-3 portions/week (150-200 g), including oily fish** **EPA+DHA: 250-500 mg/day**
Australian & New Zealand Health Authorities (333)	**Men (19+ years)**	**LC n-3 PUFA: 160 mg/day**
	Men to lower chronic disease risk	**LC n-3 PUFA: 610 mg/day**
	Women (19+ years)	**LC n-3 PUFA: 90 mg/day**
	Women to lower chronic disease risk	**LC n-3 PUFA: 430 mg/day**
European Food Safety Authority (EFSA) (334)	**General population**	**EPA+DHA: 250 mg/day**
2016 European Guidelines on cardiovascular disease prevention in clinical practice: Sixth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (335)		**Fish intake recommendations removed from guidelines.** **Reason: "With currently available cardioprotective therapies, it debatable whether EPA+DHA exert a favourable effect on all-cause, coronary artery disease and stroke mortality."**
French Food Safety Agency (AFFSA) (336)	**General population**	**EPA+DHA: 500 mg/day** **EPA: 250 mg/day** **DHA: 250 mg/day**
	Metabolic syndrome/diabetes/obesity risk reduction	**EPA+DHA: 500 mg/day**
	Cardiovascular risk reduction	**EPA+DHA: 500-750 mg/day**
Superior Health Council of Belgium (337)	**General population (primary prevention of CVD)**	**Fish intake: 2 portions/week (preferably oily fish)**
	Patients with CVD (Secondary prevention)	**EPA+DHA: 1 g/day (from fatty fish or alternatively from fish oil capsules)**
Health Council of the Netherlands (338)	**General population**	**Fish intake: One portion/week (preferably oily fish)**
Nordic Council of Ministers (339)	**General population**	**LC n-3 PUFA: at least 1% total energy intake**
Scientific Advisory Committee on Nutrition (160)	**General population**	**Fish intake: At least two portions/week, of which one should be oily** **LC n-3 PUFA: 0.45 g/day**
National Institute for Health and Clinical	**Adults at high risk of or with CVD**	**Fish intake: at least 2 portions per week, including a portion of oily fish**

Excellence (340)		**Do not offer LC n-3 PUFA supplements for prevention of CVD.**
Irish Heart Foundation (341)	**General population**	**LC n-3 PUFA: 200 mg/day**
Brazilian Society of Cardiology (342)	**Prevention of CVD**	**LC n-3 PUFA supplementation not recommended**
Academy of Nutrition and Dietetics (343)	**General population**	**EPA+DHA: 500 mg/day**
American Heart Association (344)	**Primary prevention of CHD**	**No recommendation**
	Patients with CHD (secondary prevention)	**EPA+DHA: 1 g/day (preferably from oily fish)** **EPA+DHA supplements could be considered in consultation with physician**
	Prevention of CVD mortality in patients with diabetes	**LC n-3 PUFA supplementation not indicated**
	Prevention of CHD among patients at high risk of CVD	**LC n-3 PUFA supplementation not indicated**
	Primary prevention of atrial fibrillation	**No recommendation**
	Secondary prevention of atrial fibrillation	**LC n-3 PUFA supplementation not indicated**
Health Canada (345)	**General population**	**Fish intake: At least two portions/week (provides 0.3-0.45 g/day EPA+DHA)**
American Dietetics Association / Dietitians of Canada (346)	**General population**	**LC n-3 PUFA: 500 mg/day**
Ministry of Health, Labour and Welfare, Japan (347)	**General population**	**Men (18-29 y): 2.0 g/day** **Women (18-29 y): 1.6 g/day** **Men (30-49 y): 2.1 g/day** **Women (30-49 y): 1.6 g/day** **Men (50-69 y): 2.4 g/day** **Women (50-69 y): 2.0 g/day** **Men (70+ y): 2.2 g/day** **Women (70+ y): 1.9 g/day**

Appendix 2 - Participant information sheet for the Om3ga study.

BDM/12/13-84

KING'S College LONDON

INFORMATION SHEET FOR PARTICIPANTS

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

Pilot observational study to compare heart rate variability in vegans and age-matched omnivore controls

We would like to invite you to participate in this original research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the purpose of this study?

The omega-3 fatty acids (fatty acids are the constituents of fat) are found mainly in fish, where they are derived from marine plants, but also in meat and eggs in small amounts. They are of great interest for study as a dietary intake of these omega-3 fatty acids has been proposed to influence heart rate variability (measure of heart function). Previous research has shown that vegans whose diet lack omega-3 fatty acids have only a third of the level of omega-3 fatty acids in the blood fats compared to omnivores. This research project is a pilot study and aims to investigate if there are differences in heart rate variability between vegans and omnivores. The results of this study will provide further evidence that may be used to further investigate any significant findings.

Why have I been chosen?

You have been contacted as you have expressed an interest in our research. In order to participate in this study you need to be able to answer 'Yes' to the following questions:

- I am aged between 40 and 70 years
- I do not smoke
- I have never had a heart attack, stroke, angina, pacemaker, hepatitis (liver disease), diabetes mellitus, chronic gastrointestinal disorder (celiac disease, Crohn's disease) or cancer (excluding basal cell carcinoma).
- I do not take omega-3 supplements or food fortified with omega-3 fatty acids
- I am not pregnant
- I do not take medication to lower blood pressure
- I only drink alcohol occasionally and in moderation (less than 3 to 4 drinks per day)

What will happen to me if I take part?

If you answered 'yes' to the above questions and remain interested in participating, we will ask some further questions about your health via telephone or e-mail (5 minutes). If we think you are suitable for the study we will invite you to attend a clinic appointment in the morning (around 2 hours long). We will ask you to avoid eating and drinking (except water) after 10.00pm the previous evening until attending the clinic visit the following morning between 8.00 and 11.00am. You will also be asked to complete a food frequency questionnaire prior to the visit.

On arrival, the study will be explained in detail and you will have the opportunity to ask any questions to ensure you will be giving fully informed consent. Once consent is provided, your height, weight, waist circumference and body fat measurements will be recorded and the food frequency questionnaire will be checked. We will measure your blood pressure and take a small blood sample (20 ml - about 4 teaspoons) from your arm. We will measure your blood count, liver function, blood lipid profile, vitamin B12 and glucose levels.

We will then fit a small monitoring device (Actiheart) that records heart rate and physical activity in one combined, light-weight (less than 10g) waterproof unit. You will be required to wear it for the following 24 hours. During the recording period we shall ask you to keep a record of your daily activities (activities/exercise, meals or naps). After 24h you will be asked to return the device by post using a prepaid padded enveloped or by courier. The clinic visit will take place in the Metabolic Research Unit on

4th Floor, Corridor B, Franklin-Wilkins Building, Stamford Street, SE1 9NH. We will reimburse the cost of your travel. After the clinic visit is completed you will be served a light breakfast.

Are there any risks associated with taking part in the study?

Venepuncture (taking a blood sample) may cause brief discomfort and there is a risk of bruising. The electrodes placed on the chest skin in order to fit the Actiheart monitor may cause an allergic cutaneous reaction.

Will my participation be kept confidential?

All personal information collected during the study will be kept strictly confidential. You will be provided with the results of the analysis and a letter addressed to your GP detailing results and drawing attention to any out of range values when necessary. Your GP will not be told that you are taking part in the study, unless you request us to do so. Subject confidentiality and anonymity will be maintained throughout the study by use of subject codes in place of names and the storage of subject details in a secure place. Only the investigators have access to this data and only anonymised data will be shared with other researchers. Should you wish to find out the results of this study you are welcome to contact Ana Pinto (details below) for a copy of the final report once the study is finished.

What will happen to my study results?

The study results will be presented in a report and will be published in a scientific journal. You will not be identified in the results of the study or any publication that might arise from this study. We will be happy to discuss the results with you when the study is completed, and will let you know how you can get a copy of the published results if you wish.

Who is organising and funding the study?

The study is organised by the Diabetes and Nutritional Sciences Division, Kings College London and funded by Kings College London.

Do I have to take part?

It is up to you to decide whether to take part. If you do decide to take part, you will be given this information sheet to keep and asked to sign a consent form. You can withdraw from the study at any time by informing one of the researchers and you are not obliged to give a reason. If you decide to withdraw once the study has started, the data may be used in the final report unless you request withdrawal of the data. Data cannot be withdrawn once the study has been submitted as a study report, which will be on the 9th of August.
If you decide to take part, please let us know if you have been involved in any other study in the previous year.

If this study has harmed you in any way you can contact King's College London using the details below:

Professor Tom Sanders (tom.sanders@kcl.ac.uk)

Diabetes and Nutritional Sciences Division, King's College London, Franklin Wilkins Building, 150 Stamford Street, London SE1 9NH

Thank you for your interest.

For further information, please contact:
Ana Pinto or Catherine Kidd (tel 020 7848 4162)
Email: om3gastudy@yahoo.co.uk

Address: Diabetes and Nutritional Sciences Division, King's College London, Franklin Wilkins Building
150 Stamford Street, London SE1 9NH

Appendix 3 - Activity diary given to participants in the Om3ga study.

1. PHYSICAL ACTIVITY DIARY

HOW TO FILL IN THE DIARY?

The activity diary should be completed for the recording period with the Actiheart monitor.

1. Fill in the date at the top of the page
2. For each half an hour write down the main activities you were doing and where they were done (street, work, home, gym)
3. Also register the meals, naps and sleeping times
4. Circle yes or know for each activity you have done for longer than 10 minutes without stopping

EXAMPLE **Participant code: _EX000_**

Time	Description of activity	Where?	At least 10 minutes of activity at a time without stopping?
8.00 – 8.30 AM	Walking	Street	(Yes) / No
8.30 – 9.00 AM	Sitting	Bus	Yes / No
9.00 – 9.30 AM	Walking	Street	(Yes) / No
9.30 – 10.00AM	Sitting (morning snack)	Work	Yes / No
10.00 – 10.30 AM	Sitting	Work	Yes / No
10.30 – 11.00 AM	Sitting	Work	Yes / No
11.00 – 11.30 AM	Standing	Work	Yes / (No)
11.30 – 12.00 PM	Sitting	Work	Yes / No
12.00 – 12.30 PM	Walking/Sitting (lunch)	To the cafeteria/ Cafeteria	Yes / (No)
12.30 – 1.00 PM	Strolling/Shopping	Street	Yes / (No)
1.00 – 1.30 PM	Strolling/Shopping	Street	Yes / (No)
1.30 – 2.00 PM	Sitting	Work	Yes / No
2.00 – 2.30 PM	Sitting	Work	Yes / No
2.30 – 3.00 PM	Sitting	Work	Yes / No
3.00 – 3.30 PM	Sitting	Work	Yes / No
3.30 – 4.00 PM	Walking (afternoon snack)	Street	(Yes) / No
4.00 – 4.30 PM	Zumba class	Gym	(Yes) / No
5.00 – 5.30 PM	Zumba class	Gym	(Yes) / No
5.30 – 6.00 PM	Walking/Sitting	Street/Bus	Yes / (No)
6.00 – 6.30 PM	Sitting	Train	Yes / No
6.30 – 7.00 PM	Walking/standing	Street/Home	(Yes) / No
7.00 – 7.30 PM	Standing/Sitting (dinner)	Home	Yes / (No)
7.30 – 8.00 PM	Sitting	Home	Yes / No
8.00 – 8.30 PM	Watching TV	Home	Yes / No
8.30 – 9.00 PM	Watching TV	Home	Yes / No
9.00 – 9.30 PM	Browsing the internet	Home	Yes / No
9.30 – 10.00 PM	Reading (Snack)	Home	Yes / No
10.00 – 10.30 PM	Sleeping	Home	Yes / No

Date - __/__/____ Participant code: _____

BDM/12/13-84

Time	Activity	Where?	At least 10 minutes at a time without stopping?
8.30 – 9.00 AM			Yes / No
9.00 – 9.30 AM			Yes / No
9.30 – 10.00AM			Yes / No
10.00 – 10.30 AM			Yes / No
10.30 – 11.00 AM			Yes / No
11.00 – 11.30 AM			Yes / No
11.30 – 12.00 PM			Yes / No
12.00 – 12.30 PM			Yes / No
12.30 – 1.00 PM			Yes / No
1.00 – 1.30 PM			Yes / No
1.30 – 2.00 PM			Yes / No
2.00 – 2.30 PM			Yes / No
2.30 – 3.00 PM			Yes / No
3.00 – 3.30 PM			Yes / No
3.30 – 4.00 PM			Yes / No
4.00 – 4.30 PM			Yes / No
4.30 – 5.00 PM			Yes / No
5.00 – 5.30 PM			Yes / No
5.30 – 6.00 PM			Yes / No
6.00 – 6.30 PM			Yes / No
6.30 – 7.00 PM			Yes / No
7.00 – 7.30 PM			Yes / No
7.30 – 8.00 PM			Yes / No
8.00 – 8.30 PM			Yes / No
8.30 – 9.00 PM			Yes / No
9.00 – 9.30 PM			Yes / No
9.30 – 10.00 PM			Yes / No
10.00 – 10.30 PM			Yes / No
10.30 – 11.00 PM			Yes / No
11.00 – 11.30 PM			Yes / No
11.30 – 12.00 AM			Yes / No
12.00 – 12.30 AM			Yes / No
12.30 – 1.00AM			Yes / No
1.00 – 1.30 AM			Yes / No
1.30 – 2.00 AM			Yes / No
2.00 – 2.30 AM			Yes / No
2.30 – 3.00 AM			Yes / No
3.00 – 3.30 AM			Yes / No
3.30 – 4.00 AM			Yes / No
4.00 – 4.30 AM			Yes / No
4.30 – 5.00 AM			Yes / No
5.00 – 5.30 AM			Yes / No
5.30 – 6.00 AM			Yes / No
6.00 – 6.30 AM			Yes / No
6.30 – 7.00 AM			Yes / No
7.00 – 7.30 AM			Yes / No
7.30 – 8.00 AM			Yes / No
8.00 – 8.30 AM			Yes / No
8.30 – 9.00 AM			Yes / No

Appendix 4 - EPIC-Norfolk FFQ, pages 2 to 10 used in the Om3ga and FISHH studies.

Please complete this section before going to question 1.

Date of birth: ☐ day ☐ month ☐ year

Are you male or female? Male ☐ Female ☐

How tall are you? ☐ feet and ☐ inches **or** ☐ centimetres

How much do you weigh? ☐ stones and ☐ pounds **or** ☐ kilogrammes

How old were you when you left school? ☐ years old

Do you eat any meat (including bacon, ham, poultry, game, meat pies, sausages)? Yes ☐ No ☐

If no, how old were you when you last ate meat? ☐ years old

Do you eat any fish? Yes ☐ No ☐

If no, how old were you when you last ate fish? ☐ years old

Do you eat any dairy products (including milk, cheese, butter, yogurt)? Yes ☐ No ☐

If no, how old were you when you last ate dairy products? ☐ years old

Do you eat any eggs (including eggs in cakes and other baked foods)? Yes ☐ No ☐

If no, how old were you when you last ate eggs? ☐ years old

Listed below are 130 food items divided into sections according to food type. For each food there is an amount shown, either a "medium serving" or a common household unit such as a slice or teaspoon. Please put a tick (✓) in the box to indicate how often, **on average**, you have eaten the specified amount of each food **during the last 12 months**.

EXAMPLES:

For white bread the amount is one slice, so if you ate 4 or 5 slices a day, you should put a tick in the column headed "4-5 per day".

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
BREAD AND SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls								✓	

For chips, the amount is a "medium serving", so if you had a helping of chips twice a week you should put a tick in the column headed "2-4 per week".

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
POTATOES, RICE AND PASTA (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Chips				✓					

For very seasonal fruits such as strawberries and raspberries you should estimate your average use when the fruits are in season, so if you ate strawberries or raspberries about once a week when they were in ries, kiwi fruit ✓

1. Please estimate your average food use as best you can, and please answer every question.

MEAT AND FISH

Did you eat any meat or fish in the last 12 months? Yes ☐ No ☐

If no, please go to next page

If yes, please fill in this page

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
MEAT AND FISH (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: roast, steak, mince, stew or casserole									
Beefburgers									
Pork: roast, chops, stew or slices									
Lamb: roast, chops or stew									
Chicken or other poultry e.g. turkey									
Bacon									
Ham									
Corned beef, Spam, luncheon meats									
Sausages									
Savoury pies, e.g. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls									
Liver, liver pate, liver sausage									
Fried fish in batter, as in fish and chips									
Fish fingers, fish cakes									
Other white fish, fresh or frozen, e.g. cod, haddock, plaice, sole, halibut									
Oily fish, fresh or canned, e.g. mackerel, kippers, tuna, salmon, sardines, herring									
Shellfish, e.g. crab, prawns, mussels									
Fish roe, taramasalata									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

What did you do with the visible fat on your meat?

Ate most of the fat ☐ Ate as little as possible ☐

Ate some of the fat ☐ Did not eat meat ☐

How often did you eat grilled or roast meat? ☐☐ times a week

How well cooked did you usually have grilled or roast meat?

Well done /dark brown ☐ Lightly cooked/rare ☐

Medium ☐ Did not eat meat ☐

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
BREAD AND SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls									
Brown bread and rolls									
Wholemeal bread and rolls									
Cream crackers, cheese biscuits									
Crispbread, e.g. Ryvita									
CEREALS (one bowl)									
Porridge, Readybrek									
Breakfast cereal such as cornflakes, muesli etc.									
POTATOES, RICE AND PASTA (medium serving)									
Boiled, mashed, instant or jacket potatoes									
Chips									
Roast potatoes									
Potato salad									
White rice									
Brown rice									
White or green pasta, e.g. spaghetti, macaroni, noodles									
Wholemeal pasta									
Lasagne, moussaka									
Pizza									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
DAIRY PRODUCTS AND FATS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream (tablespoon)									
Double or clotted cream (tablespoon)									
Low fat yogurt, fromage frais (125g carton)									
Full fat or Greek yogurt (125g carton)									
Dairy desserts (125g carton)									
Cheese, e.g. Cheddar, Brie, Edam (medium serving)									
Cottage cheese, low fat soft cheese (medium serving)									
Eggs as boiled, fried, scrambled, etc. (one)									
Quiche (medium serving)									
Low calorie, low fat salad cream (tablespoon)									
Salad cream, mayonnaise (tablespoon)									
French dressing (tablespoon)									
Other salad dressing (tablespoon)									
The following on bread or vegetables									
Butter (teaspoon)									
Block margarine, wrapped, NOT tub, e.g. Stork,Krona (teaspoon)									
Polyunsaturated margarine, in tub, e.g. Flora, sunflower (teaspoon)									
Other soft margarine, dairy spreads, in tub, e.g. Blue Band, Clover (teaspoon)									
Low fat spread, in tub, e.g. Outline, Gold (teaspoon)									
Very low fat spread, in tub (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
SWEETS AND SNACKS (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Sweet biscuits, chocolate, e.g. digestive (one)									
Sweet biscuits, plain, e.g. Nice, ginger (one)									
Cakes e.g. fruit, sponge, home baked									
Cakes e.g. fruit, sponge, ready made									
Buns, pastries e.g. scones, flapjacks, home baked									
Buns, pastries e.g. croissants, doughnuts, ready made									
Fruit pies, tarts, crumbles, home baked									
Fruit pies, tarts, crumbles, ready made									
Sponge puddings, home baked									
Sponge puddings, ready made									
Milk puddings, e.g. rice, custard, trifle									
Ice cream, choc ices									
Chocolates, single or squares									
Chocolate snack bars e.g. Mars, Crunchie									
Sweets, toffees, mints									
Sugar added to tea, coffee, cereal (teaspoon)									
Crisps or other packet snacks, e.g. Wotsits									
Peanuts or other nuts									
SOUPS, SAUCES, AND SPREADS									
Vegetable soups (bowl)									
Meat soups (bowl)									
Sauces, e.g. white sauce, cheese sauce, gravy (tablespoon)									
Tomato ketchup (tablespoon)									
Pickles, chutney (tablespoon)									
Marmite, Bovril (teaspoon)									
Jam, marmalade, honey (teaspoon)									
Peanut butter (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
DRINKS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tea (cup)									
Coffee, instant or ground (cup)									
Coffee, decaffeinated (cup)									
Coffee whitener, e.g. Coffee-mate (teaspoon)									
Cocoa, hot chocolate (cup)									
Horlicks, Ovaltine (cup)									
Wine (glass)									
Beer, lager or cider (half pint)									
Port, sherry, vermouth, liqueurs (glass)									
Spirits, e.g. gin, brandy, whisky, vodka (single)									
Low calorie or diet fizzy soft drinks (glass)									
Fizzy soft drinks, e.g. Coca cola, lemonade (glass)									
Pure fruit juice (100%) e.g. orange, apple juice (glass)									
Fruit squash or cordial (glass)									
FRUIT (1 fruit or medium serving) **For very seasonal fruits such as strawberries, please estimate your average use when the fruit is in season**									
Apples									
Pears									
Oranges, satsumas, mandarins									
Grapefruit									
Bananas									
Grapes									
Melon									
Peaches, plums, apricots									
Strawberries, raspberries, kiwi fruit									
Tinned fruit									
Dried fruit, e.g. raisins, prunes									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
VEGETABLES Fresh, frozen or tinned (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Carrots									
Spinach									
Broccoli, spring greens, kale									
Brussels sprouts									
Cabbage									
Peas									
Green beans, broad beans, runner beans									
Marrow, courgettes									
Cauliflower									
Parsnips, turnips, swedes									
Leeks									
Onions									
Garlic									
Mushrooms									
Sweet peppers									
Beansprouts									
Green salad, lettuce, cucumber, celery									
Watercress									
Tomatoes									
Sweetcorn									
Beetroot									
Coleslaw									
Avocado									
Baked beans									
Dried lentils, beans, peas									
Tofu , soya meat, TVP, Vegeburger									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

Your diet last year, continued

2. Are there any **other** foods which you ate more than once a week? Yes ☐ No ☐

If yes, please list below

Food	Usual serving size	Number of times eaten each week

3. What type of milk did you most often use?

Select one only

Full cream, silver ☐ Semi-skimmed, red/white ☐
Skimmed/fat free ☐ Channel Islands, gold ☐
Dried milk ☐ Soya ☐
Other ☐ specify ☐ None ☐

If you used soya milk, please describe brand and type ☐

4. How much milk did you drink each day, including milk with tea, coffee, cereals etc?

None ☐ Three quarters of a pint ☐
Quarter of a pint ☐ One pint ☐
Half a pint ☐ More than one pint ☐

5. Did you usually eat breakfast cereal, excluding porridge and Ready Brek mentioned earlier? Yes ☐ No ☐

If yes, which brand and type of breakfast cereal, including muesli, did you usually eat?

List the one or two types most often used

Brand	Type

6. What kind of fat did you most often use for frying, roasting, grilling etc?

Select one only

Butter ☐ Solid white vegetable fat ☐
Lard/dripping ☐ Margarine ☐
Vegetable oil ☐ None ☐

If you used vegetable oil, please give type e.g. corn, sunflower ☐

7. What kind of fat did you most often use for baking cakes etc?

Select one only

Butter ☐ Solid white vegetable fat ☐
Lard/dripping ☐ Margarine ☐
Vegetable oil ☐ None ☐

If you used margarine, please give type e.g. Flora, Stork ☐

8. How often did you eat food that was fried at home?

Daily ☐ Less than once a week ☐
4-6 times a week ☐ Never ☐
1-3 times a week ☐

9. How often did you eat fried food away from home?

Daily ☐ Less than once a week ☐
4-6 times a week ☐ Never ☐
1-3 times a week ☐

10. How often did you add salt to food while cooking?

Always ☐ Rarely ☐
Usually ☐ Never ☐
Sometimes ☐

11. How often did you add salt to any food at the table?

Always ☐ Rarely ☐
Usually ☐ Never ☐
Sometimes ☐

12. Did you regularly use a salt substitute (e.g. LoSalt)? Yes ☐ No ☐

If yes, which brand? ☐

13. Have you regularly taken any vitamins, minerals, fish oils, fibre or other food supplements during the last 12 months? Yes ☐ No ☐

If yes, list brand and daily dose

Name and brand of supplements	Daily dose

14. In the last 12 months, have you eaten a modified diet for any of these reasons?

Tick more than one box if applicable

High blood pressure ☐ High blood cholesterol ☐
Stomach problems (e.g. ulcer or gastritis) ☐ Overweight/obesity ☐
Bowel problems (e.g. irritable bowel or diverticulitis) ☐ Diabetes ☐
Allergies (e.g. skin rash) ☐ Concern over eating a healthy diet ☐
Concern over a family history of illness ☐ Not modified my diet ☐
Other ☐ specify ☐

Appendix 5 - Blood handling protocol in the Om3ga study.

Sample	Tubes	Ice/RT	Centrifuge	Separation	Analytes	Storage	Transport to lab
Screening	4ml Fluoride oxalate	Ice	15 min x 1500 g at 4ºC	Separate plasma into 2 x 1 ml aliquots	Glucose	Freeze 1 aliquot send the other fresh to analyse	KCH - Same day at room temperature
	4ml EDTA	RT	No	None	FBC	RT	KCH - Same day at room temperature
	5ml SST™	RT	15 min x 1500 g at 4ºC	Separate serum into 2 x 1 ml aliquots	FLIP LFT	Freeze 1 aliquot send the other fresh to analyse	KCH - Same day at room temperature
Fatty acid analysis	6 ml EDTA	RT	15 min x 1500 g at 4ºC	***Plasma*** (top layer) - Separate into 2 x 1 ml aliquots	Plasma fatty acid composition	Freeze 1 aliquot at -80ºC	KCL – GC analysis
					Lipidomics	Freeze 1 aliquot at -80ºC	University of Manchester
				RBC (bottom layer)	Erythrocyte membrane phospholipid fatty acid composition	Store in the fridge at 4ºC	KCL – Wash RBC and freeze at - 20ºC until analysed

FBC, full blood count; FLIP, fasting lipid profile; KCH, Kings College Hospital; KCL, King's College London; LFT, liver function test; RBC, red blood cells; RT, room temperature.

Appendix 6 - Participant information sheet from the FISHH study.

King's College Hospital NHS Foundation Trust Guy's and St Thomas' NHS Foundation Trust KING'S College LONDON

INFORMATION SHEET FOR PARTICIPANTS

REC Reference Number: 14/LO/0186

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

Heart rate variability and omega-3 fatty acid status: haemodialysis patients "The FISHH Study"

We would like to invite you to participate in this original research project undertaken as part of a PhD programme. This study has been reviewed and approved by the Camberwell St Giles Research Ethics Committee. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the purpose of this study?

Regularly eating omega-3 fats, which are found mainly in oily fish, is likely to be heart-protective. Studies suggest that dietary omega-3 fats influence how much the time interval between each heart beat varies (heart rate variability; HRV). High HRV is thought to indicate that the heart is responding well. Previous research has shown patients with kidney disease have lower levels of omega-3 fats, compared to people without kidney problems. This research project will measure the variation in heart rate and amount of omega-3 fats in patients who have recently commenced haemodialysis and will determine if there is a relationship between HRV and the proportion of fats in the blood that are omega-3 fats.

Are you suitable for this study?

You have been given this information sheet as you have expressed an interest in our research. In order to participate in this study you need to be able to answer 'Yes' to all of the following:

- ✓ I am aged between 40 and 80 years
- ✓ I have recently started haemodialysis treatment (up to 10 weeks)
- ✓ I haven't had any infection or been on antibiotics for the last month
- ✓ I don't have a history of chronic liver disease or neuropathy (abnormal nerve function)

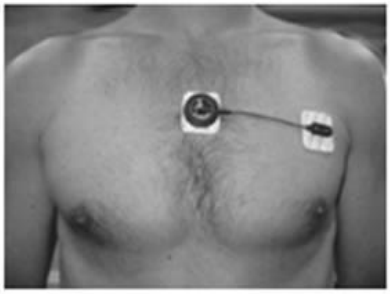
What will happen to you if you take part?

If you answered 'yes' to the above questions and remain interested in participating, we will ask some further questions about your health over a clinic visit with Dr. Helen MacLaughlin (Lead Renal Dietitian) at the hospital. During this first visit, the study will be explained in

detail and you will have the opportunity to ask any questions to ensure you will be giving fully informed consent. If you would like to participate in the study, we will then ask you to sign a consent form. Eligible participants will be invited to take part in the study and a meeting with Ana Pinto (PhD student) will be arranged for the same day of a mid-week dialysis session (Wednesday or Thursday). During this visit you will still have the opportunity to ask any questions about the study.

At the same visit, a small pre dialysis blood sample (20 ml – about 4 teaspoons) and post dialysis blood sample (10ml – about 2 teaspoons) will be taken by a nurse and used to measure omega-3 fats, standard dialysis renal blood tests (electrolytes) and C-reactive protein (a marker of inflammation). During the dialysis session, we will then fit a small monitoring device (Actiheart) on your chest that records heart rate and physical activity (Figure 1). You will be required to wear it for 24 hours, then remove it yourself, and return it at your next haemodialysis session. During the recording period we shall ask you to keep a diary of duration of dialysis, activity levels for the rest of the day, and sleep times at night. You will also be asked to complete a dietary questionnaire, to provide information on your habitual diet, and to respond to a sleep questionnaire. Participants will receive standard care as normal.

Figure 1 – Actiheart monitor placed on the chest.

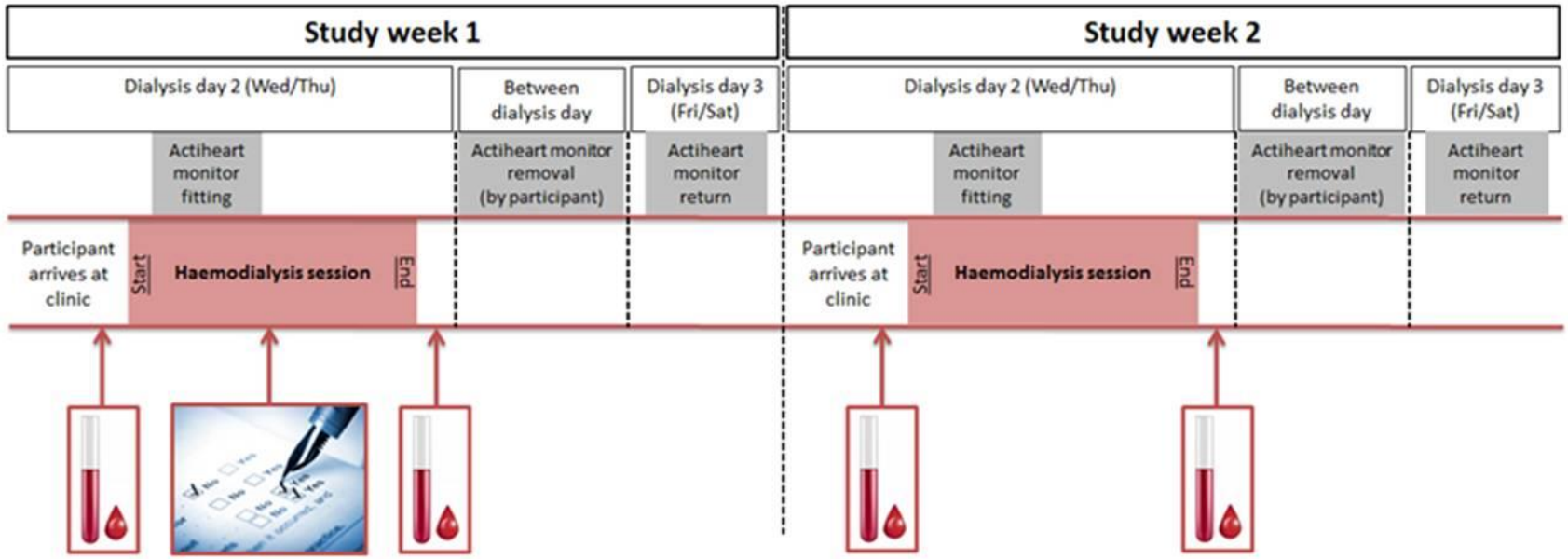


We will need to obtain details about your medical history including pre-dialysis blood test results, medication history, blood pressure and complications from your medical notes.

After one to two weeks, a second visit (again on a Wednesday or Thrusday during your usual dialysis session) will include another small blood sample (20 ml – about 4 teaspoons) prior to the dialysis session taken by a nurse. We will fit the Actiheart monitor a second time for another 24h recording and you will need to return it at your next haemodialysis session. We are doing measurements in duplicate to see how close the measures are to each other and if there are any differences. Neither study days will require any extra time from you as all blood sampling and monitor fitting will take place during your usual dialysis session.

Should you become unable to provide consent during the study, you will not be asked to wear the Actiheart monitor or to complete the questionnaires. However, in the event of loss of capacity to provide consent we will still retain any data/blood sample collected up to that point.

The overall study design is shown in the diagram below.



Are there any risks associated with taking part in the study?
The pads placed on the chest skin, under the Actiheart monitor, may cause minor irritation to the skin and in a few cases, may cause an allergic reaction, leaving a temporary rash.

Will your participation be kept confidential?
Information collected about you during this research will be kept strictly confidential. Your nephrologist will be informed of any abnormal haemodialysis blood test results. Your GP will not be told that you are taking part in the study, unless you would like us to do so. Patient confidentiality and anonymity will be maintained throughout the study by use of identification codes in place of names, and the storage of subject details in a secure place. Only investigators have access to this data and only anonymised data will be shared with other researchers. Should you wish to find out the results of this study you are welcome to contact Ana Pinto (details below) for a copy of the final report once the study is finished.

What will happen to your study results?
The study results will be presented in a report and we hope to publish them in a scientific journal. You will not be identified in the results of the study or any publication that might arise from this study. We will be happy to discuss the overall results with you when the study is completed, and will let you know how you can get a copy of the published results if you wish.

Who is organising and funding the study?
The study is organised by the Diabetes and Nutritional Sciences Division, School of Medicine, King's College London and is funded by King's College London

Do you have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You can withdraw from the study at any time by informing one of the researchers and you are not obliged to give a reason. If you decide to withdraw once the study has started, the data may be used in the final report unless you request withdrawal of the data. You can withdraw your data from the study up until the time of publication (December 2014). A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.
If you decide to take part, please let us know if you have been involved in any other study in the last year.

Thank you for your interest.
For further information, please contact:
Ana Pinto by email on FISHHstudy@kcl.ac.uk or by telephone on 020 7848 4162.
Helen MacLaughlin (Lead Renal Dietitian) by telephone on 020 3299 6250.
If this study has harmed you in any way you can contact King's College London using the details below:

Dr. Wendy Hall (wendy.hall@kcl.ac.uk)

Diabetes and Nutritional Sciences Division, King's College London, Franklin Wilkins Building, 150 Stamford Street, London SE1 9NH

Telephone: 020 7848 4197 or 020 7848 4162

Appendix 7 - Activity and event diary for participants in the FISHH study.

King's College Hospital NHS Foundation Trust | Guy's and St Thomas' NHS Foundation Trust | King's College London

1. ACTIVITY AND EVENT DIARY

HOW TO FILL IN THE DIARY?

The activity diary should be completed for the recording period with the Actiheart monitor.

1. Fill in the date at the top of the page
2. For each half an hour write down the main activities you were doing and where they were done (street, work, home, gym)
3. Also register the meals, naps and estimate the time you went to sleep and the time you woke up
4. Write in the observations column any emotional feelings you experienced

EXAMPLE **Participant code: _EX000_**

Time	Description of activity	Where?	Observations
8.00 – 8.30 AM	Sitting/Standing	Home	Woke up around 8.00
8.30 – 9.00 AM	Sitting/Standing	Home	Breakfast
9.00 – 9.30 AM	Sitting	Home	
9.30 – 10.00AM	Sitting	Home	
10.00 – 10.30 AM	Sitting	Home	
10.30 – 11.00 AM	Sitting/Standing	Home	morning snack
11.00 – 11.30 AM	Sitting	Home	
11.30 – 12.00 PM	Sitting	Home	
12.00 – 12.30 PM	Walking/Sitting	To the restaurant/ restaurant	5 minute walk lunch
12.30 – 1.00 PM	Strolling/Shopping	Street	
1.00 – 1.30 PM	Strolling/Shopping	Street	
1.30 – 2.00 PM	Sitting	Home	
2.00 – 2.30 PM	Sitting	Home	
2.30 – 3.00 PM	Walking	Street	15 minute walk
3.00 – 3.30 PM	Sitting	Friend's house	
3.30 – 4.00 PM	Sitting	Friend's house	afternoon snack
4.00 – 4.30 PM	Playing chess	Friend's house	Felt anxious
5.00 – 5.30 PM	Playing chess	Friend's house	Felt anxious
5.30 – 6.00 PM	Walking	Street	15 minute walk
6.00 – 6.30 PM	Standing/Sitting	Home	
6.30 – 7.00 PM	Standing/Sitting	Home	
7.00 – 7.30 PM	Standing/Sitting	Home	dinner
7.30 – 8.00 PM	Sitting	Home	
8.00 – 8.30 PM	Watching TV	Home	
8.30 – 9.00 PM	Watching TV	Home	
9.00 – 9.30 PM	Browsing the internet	Home	
9.30 – 10.00 PM	Reading	Home	Snack
10.00 – 10.30 PM	Sleeping	Home	Went to sleep around 10.20

Date - __/__/____ **Participant code: _____**

Time	Activity	Where?	Observations
8.30 – 9.00 AM			
9.00 – 9.30 AM			
9.30 – 10.00AM			
10.00 – 10.30 AM			
10.30 – 11.00 AM			
11.00 – 11.30 AM			
11.30 – 12.00 PM			
12.00 – 12.30 PM			
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6.30 – 7.00 AM			
7.00 – 7.30 AM			
7.30 – 8.00 AM			
8.00 – 8.30 AM			
8.30 – 9.00 AM			

Appendix 8 - Epworth sleepiness scale and Berlin questionnaire used in the FISHH study.

King's College Hospital NHS Foundation Trust Guy's and St Thomas' NHS Foundation Trust KING'S College LONDON

REC Reference Number: 14/LO/0186

Participant ID: Date: ____/____/_____

Height: _____ Weight (kg): _____ Neck (cm): _____

EPWORTH SLEEPING SCALE

The following questionnaire will help you measure your general level of daytime sleepiness. Please fill in the questionnaire by filling in a number for each situation using the following scale:

"How likely are you to doze off or fall asleep?"

0 = would never doze 2 = moderate chance of dozing

1 = slight chance of dozing 3 = high chance of dozing

It is important that you circle a number (0 to 3) for EACH situation.

Sitting reading a book	0 1 2 3
Watching television	0 1 2 3
Sitting inactive in a public place (eg. Theatre/meeting)	0 1 2 3
Lying down to rest in the afternoon	0 1 2 3
Sitting talking to someone	0 1 2 3
Sitting quietly after a lunch without alcohol	0 1 2 3
In a car, stopped in traffic or at lights	0 1 2 3
In a car, as a passenger for an hour	0 1 2 3
TOTAL SCORE	

BERLIN QUESTIONNAIRE	
Category 1	
1. Has your weight changed?	***a) Increased*** b) Decreased c) No change
2. Do you snore?	***a) Yes*** b) No c) Don't know
3. Snoring loudness	a) Loud as breathing b) Loud as talking ***c) Louder than talking*** d) ***Very loud***
4. How often do you snore?	***a) Nearly everyday*** ***b) 3 - 4 times a week*** c) 1 - 2 times a week d) Never or nearly never
5. Has you snoring ever bother other people?	***a) Yes*** b) No c) Don't know
6. How often have your breathing pauses been noticed?	***a) Nearly every day*** ***b) 3 - 4 times a week*** c) 1 - 2 times a week d) 1 - 2 times a month e) Never or nearly never
Category 2	
7. How often are you tired or fatigued after sleeping?	***a) Nearly every day*** ***b) 3 - 4 times a week*** c) 1 - 2 times a week d) Never or nearly never
8. During your waking time, do you feel tired, fatigued or not up to par?	***a) Nearly every day*** b) ***3 - 4 times a week*** c) 1 - 2 times a week d) Never or nearly never
9. Have you ever nodded off or fallen asleep while driving a vehicle?	***a) Yes*** b) No
Category 3	
10. Do you have high blood pressure?	***a) Yes*** b) No
BMI	=

Appendix 9 - Participant information sheet for the Met-IER study.

INFORMATION SHEET FOR PARTICIPANTS

KCL Ethics Ref: HR-15/16-2179

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

KING'S College LONDON

A randomised controlled trial assessing the impact of an intermittent energy restricted diet on weight loss, insulin sensitivity and heart rate variability in healthy men and women with central obesity. "The Met-IER Study"

We would like to invite you to participate in this original research project undertaken as part of a PhD programme. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the purpose of the study?

Daily calorie restriction diets are the main approach used for weight loss. We would like to find out if intermittent calorie-restricted diets (a modified "5:2 diet") can improve markers of health, for example, blood sugar levels and blood pressure. We want to compare this modified 5:2 diet (consisting of 2 days of very low calorie intakes followed by 5 days of less restricted calorie intake for 4 weeks), to a standard daily calorie-restricted diet to see if there are additional benefits for heart health and blood sugar levels with the 5:2 approach.

Why have I been invited to take part?

You have been invited as you have expressed an interest in our research.
We would like to study 34 men and women who can answer 'Yes' to the following questions:

- I am aged between 35 and 75 years
- Waist measurements are greater than the following cut-offs:
    a) **I am female,** identify as having a **South Asian or East Asian** ethnic background, and my waist measures more than 80 cm (32 inches) ***OR,***
    b) **I am female,** identify as **Black African/Black Caribbean, White, Mixed/Multiple Ethnic groups, or other** ethnic group, and my waist measures more than 88 cm (35 inches), ***OR,***
    c) **I am male,** identify as having a **South Asian or East Asian** ethnic background, and my waist measures more than 90 cm (36 inches) ***OR,***
    d) **I am male,** identify as **Black African/Black Caribbean, White, Mixed/Multiple Ethnic groups, or other** ethnic group, and my waist measures more than 102 cm (41 inches).
- I do not smoke

- I have never had a heart attack, stroke, angina, liver or kidney disease, diabetes mellitus, chronic gastrointestinal disorder (celiac disease, Crohn's disease) or cancer (excluding basal cell carcinoma).
- I do not have uncontrolled epilepsy or uncontrolled depression
- I have not had bariatric surgery or other major surgery
- I have not participated in a weight management drug trial in the previous 3 months
- I am not pregnant, lactating or planning pregnancy within the study period
- I do not take medication to lower my weight
- I do not have a history of excess alcohol intake or substance abuse

Do I have to take part?

No. It is up to you to decide whether to take part. If you do decide to take part, we will go through this information sheet with you and answer any questions you may have. You will be given this information sheet to keep and asked to sign 2 copies of a consent form. You can withdraw from the study at any time by informing one of the researchers and you are not obliged to give a reason. If you decide to withdraw once the study has started, the data may be used in the final report unless you request withdrawal of the data. Data cannot be withdrawn once the study has been submitted as a study report, which will be on the **1st of September 2016**. If you decide to take part, please let us know if you have been involved in any other study in the previous year.

What will happen to me if I take part?

If you answer 'yes' to the above questions and remain interested in participating, we will ask some further questions about your health via telephone or email (approx. 10 mins). You will be invited to a screening visit to confirm eligibility, and then you will be enrolled on the study. The study will last 4-5 weeks and you will attend King's College London on 3 occasions to provide information about your diet and mood, have your weight and body fat measured, give blood samples, undergo some tests on your blood vessels and carry out a computer based task. Each of these visits will last up to 3 h 15 min. You will also be offered the chance to attend on 2 further occasions for group support sessions (30 min) to help you with sticking to the diet.

Further details can be found below.

Screening visit:
If we think you are potentially suitable for the study you will be invited to attend a clinic screening appointment in the morning (approx. 45 mins) in the **Metabolic Research Unit on 4th Floor, Corridor A, Franklin-Wilkins Building, 150 Stamford Street, London, SE1 9NH** (close to Waterloo Station). We will ask you to avoid eating and drinking (except water) for 12h overnight until attending the clinic visit the following morning between 8.00 and 11.00am.

On arrival, the study will be explained in detail and you will have the opportunity to ask any questions to ensure you will be giving fully informed consent. Once consent is provided, your height, weight, waist circumference and body fat measurements will be recorded and a small fasting blood sample (11 ml - about 2 teaspoons) will be taken from your arm to measure your blood count, liver function, blood fats and glucose level and check that they are within normal ranges. We will measure seated blood pressure and you will be asked to fill in an eating behaviour questionnaire. If you are a woman, we will ask you about your menopausal status and when you last had a menstrual period. You will be provided with a 7 day food diary and instructions will be given to you on how to fill it in. A light breakfast will be provided once all samples and measurements have been made.

A copy of the screening results will be given to you upon request. If any abnormal results are found we will inform you immediately and we will provide you with a letter to give to your GP if necessary. Following screening, if your results comply with the study inclusion criteria you will be invited to attend the Metabolic Research Unit on 3 further occasions. You will also be asked to discontinue dietary therapies/supplements such as probiotics, prebiotics, fibre supplements, laxatives or any other constipation remedies, vitamins/minerals/oils other than multivitamins providing 100% of your daily requirements from now until the end of the study.

Study visits (baseline visit, 2nd and 3rd study visits):
You will be asked to refrain from alcohol and any strenuous exercise for 24 h before each visit. We will also ask you to avoid eating or drinking (except water) for 12h overnight until the arranged time of your morning visit. You will be randomly allocated to 1 of the following 2 diets, by computer software, for a 4 week period: 1) a standard daily calorie restricted diet, or, 2) an intermittent (5:2) calorie restricted diet. We will not be able to change the diet that you have been allocated as this would lead to bias in the study results.

Diet and physical exercise advice will be given to all participants. This will be delivered in detail at visit 1, and further advice will be offered over the telephone during the study period. Group support sessions will be available to help you stick to the diet.

1) You will be asked to eat a low-fat meal in the evening before your first visit and report to the Metabolic Research Unit between 8.00 and 11.00am, in the fasted state (i.e. without having consumed breakfast and without having consumed any food or drink, expect water, for the past 12h). Make sure you drink some water on the morning of the study to avoid dehydration.
2) Visit 1 will take approximately 3 h 15 min, and visits 2 and 3 will take approximately 2h 30 min each. At each visit we will take a fasting blood sample (27.5 ml – about 5-6 teaspoons) to make measurements to assess your metabolic profile including glucose levels, different types of blood fats, markers of inflammation, substances related to appetite regulation, stress and substances produced in response to fasting.
3) Your basal metabolic rate (BMR) will be estimated, which is the energy you burn when you are at rest. This measurement will be made using a small portable device where

you will need to lie down with your head placed underneath a transparent plastic canopy for approximately 15 to 20 minutes.

4) You will be fitted with a small, lightweight heart rate monitor to be worn on the chest for 24 hours. The fitting involves skin preparation including the use of a cleansing wipe, abrasive pad (like an emery board) and shaving part of the chest hair where required. The monitor will be secured on to the skin with sticky pads. An ambulatory blood pressure monitor will be fitted so that we can make measurements of your blood pressure at intervals over a 24 hour period. A standard arm cuff will be placed around your arm and will inflate every 30 mins during the day and every hour at night. We will provide you with a diary so you can record your daily activities (activities/exercise, meals or naps) and make a note to any changes in your emotional state for the 24h you will be having the monitors on.
5) You will be asked to perform a computer based colour-word test (15 min) and a computer-based pattern recognition test (15 min).
6) You will be asked to fill in an eating habit questionnaire and a mood/emotion questionnaire.
7) You will be provided with another 7-day food diary to complete during week 3 of the study.
8) Optionally, you will be asked to provide a small stool sample, once at the beginning and twice at the end of the study. If you agree to take part in this supplementary stool analysis study, you will need to deliver a single fresh stool sample to the study centre within 3 hours of collection. This will provide us with valuable information about the bacteria in your gut and your gut health. *This is an additional measurement and if you do not want to take part in this supplementary study, you can still take part in the main study.*
9) At the end of each study visit a light breakfast will be provided.
10) The second and third study visits will take place at the end of week 4. All the above procedures and measurements will be repeated at each of the study visits (please see figure 1 for a study timeline).
11) Group support sessions, including motivational support and discussions on how to overcome challenges in sticking to the diet, will be offered at the end of week 1 and week 3.

<u>Participants allocated the daily calorie restricted (standard) diet:</u>

You will be provided with dietary and exercise advice and a personalised dietary plan on how to reduce your calorie intake by 500 calories per day for 4 weeks.

<u>Participants allocated the intermittent calorie restricted (5:2) diet:</u>

You will be provided with dietary and exercise advice on how to follow a very low calorie diet (VLCD) on 2 consecutive days alternated with a normal healthy diet with a mild calorie restriction for 5 consecutive days, for four weeks. The VLCD will be provided as meal replacement food packs, supplied by Lighterlife®. You can choose which days of the week

to follow the VLCD, but they must be two successive days, e.g. Monday and Tuesday, or Wednesday and Thursday.

Incentives (where relevant)

Participants will be paid £75 upon completion of the 3 study days. Travel expenses will be reimbursed up to £10 per screening/group support/study day.

What are the possible benefits and risks of taking part?

Benefits: You will receive information about your health (blood pressure, blood fats, sugars, liver function and blood cells) and diet and lifestyle advice from nutrition students. When you lose weight you may feel an improvement in your general wellbeing.
Risks: Venepuncture (taking a blood sample) may cause brief discomfort and there is a risk of bruising. The electrodes placed on the chest skin in order to fit the heart monitor may very rarely cause a minor allergic skin reaction and the cuff used to measure blood pressure may cause a tingling sensation in the arm. In the unlikely event we discover that you have a medical condition that you did not know about before, we will tell you about it and discuss how this should be dealt with.

Will my taking part be kept confidential?

All personal information collected during the study will be kept strictly confidential. You will be provided with the results of the analysis and a letter addressed to your GP detailing results and drawing attention to any "out of range" values if necessary. Your GP will not be told that you are taking part in the study, unless you request us to do so. Subject confidentiality and anonymity will be maintained throughout the study by use of subject codes in place of names and the storage of subject details in a secure place. Only the investigators have access to this data and only anonymised data will be shared with other researchers. Should you wish to find out the results of this study you are welcome to contact Ana Pinto (details below) for a copy of the final report once the study is finished.

How is the project being funded?

The study is organised by the Diabetes and Nutritional Sciences Division, Kings College London and funded by LighterLife® (UK) Ltd.

What will happen to the results of the study?

The study results will be presented in a report and will be published in a scientific journal. You will not be identified in the results of the study or any publication that might arise from

this study. We will be happy to discuss the results with you when the study is completed, and will let you know how you can get a copy of the published results if you wish.

Who should I contact for further information?

If you have any questions or require more information about this study, please contact me using the following contact details:

Ana Pinto (tel 02078484162 or 07784498360)
Email: met-ier@kcl.ac.uk

What if I have further questions, or if something goes wrong?

If this study has harmed you in any way or if you wish to make a complaint about the conduct of the study you can contact King's College London using the details below for further advice and information:

Dr. Wendy Hall (Principal Investigator)
Senior Lecturer in Nutritional Sciences
Diabetes and Nutritional Sciences Division, King's College London, Franklin Wilkins Building, 150 Stamford Street, London SE1 9NH
wendy.hall@kcl.ac.uk

Thank you for reading this information sheet and for considering taking part in this research.

Appendix 10 - Activity diary for participants of the Met-IER study.

Activity Diary

KING'S College LONDON

Subject code .....

Please complete this activity diary during periods when the heart rate monitor is fitted and return it with the Actiheart heart rate monitor when the 24 hour recording has finished.

1. ACTIVITY DIARY
HOW TO FILL IN THE DIARY?
1. Fill in the date at the top of the page 2. For each half an hour write down the main activities you were doing and where they were done (street, work, home, gym) 3. Also register the meals, naps and estimate the time you went to sleep and the time you woke up 4. Write in the observations column any strong emotional feelings you experience (anger, sadness, nervous, anxiety, etc) 5. Write yes or no for each activity you have done non-stop for over 10 minutes

Example:

Date _22_/_01_/_2016___

Time	Description of Activity	Where?	Observations	At least 10 minutes of non-stop activity
0800 - 0830	Walking	Street		Yes
0830 - 0900	Sitting	Bus		No
0900 - 0930	Walking	Street		Yes
0930 - 1000	Sitting (snack)	Work	Stressed	No
1000 - 1030	Sitting	Work	Upset	No
1030 - 1100	Standing	Work		No
1100 - 1130	Sitting	Work		No
1130 - 1200	Sitting/Walking	Work/Street		Yes
1200 - 1230	Walk/sitting	Street	Lunch	No

Version 1.0, Dated 26/11/15
HR-15/16-2179

Date: __/__/____ Visit Number: _____ Participant code:_____

Time	Description of activity	Where?	Observations	At least 10min of non-stop activity?
8.00 – 8.30 AM				
8.30 – 9.00 AM				
9.00 – 9.30 AM				
9.30 – 10.00AM				
10.00 – 10.30 AM				
10.30 – 11.00 AM				
11.00 – 11.30 AM				
11.30 – 12.00 PM				
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12.30 – 1.00AM				

1.00 – 1.30 AM				
1.30 – 2.00 AM				
2.00 – 2.30 AM				
2.30 – 3.00 AM				
3.00 – 3.30 AM				
3.30 – 4.00 AM				
4.00 – 4.30 AM				
4.30 – 5.00 AM				
5.00 – 5.30 AM				
5.30 – 6.00 AM				
6.00 – 6.30 AM				
6.30 – 7.00 AM				
7.00 – 7.30 AM				
7.30 – 8.00 AM				
8.00 – 8.30 AM				
8.30 – 9.00 AM				
9.00 – 9.30 AM				
9.30 – 10.00AM				
10.00 – 10.30 AM				
10.30 – 11.00 AM				
11.00 – 11.30 AM				
11.30 – 12.00 PM				

Time you went to sleep:_____ **Time you woke up next day: _____**

Appendix 11 - Blood handling protocol for screening and study days in the Met-IER study.

Sample	Tubes	Centrifuge	Separation	Analytes	Storage	Amount to aliquot for KCH
Screening	2ml Fluoride oxalate	15 min x 1500 g at RT	2 x 0.5 ml	Glucose	Freeze 0.5 ml	0.5 ml
	4ml EDTA	No	None	FBC	N/A	Send sample without aliquoting
	5 ml SST™	15 min x 1500 g at RT	2 x 1 ml	FLIP LFT	Freeze 1 ml	1 ml
Study days	4ml Fluoride oxalate	15 min x 1500 g at 4ºC	4 x 0.5 ml	Glucose	Freeze 3 x 0.5 ml	0.5 ml
	6ml EDTA		0.2 ml	IL-6	-	0.2 ml
			0.8 ml	Metanephrines	-	0.8 ml
			2 x 0.5 ml	Nitrates	Freeze 2 x 0.5 ml	-
			0.6 ml	EDTA spare	Freeze 0.6 ml	-
	17ml SST™ (8.5ml x 2)		0.5 ml + 0.4 ml	FLIP	Freeze 0.4 ml	0.5 ml
			0.2 ml	Insulin	Freeze 0.2 ml	1ml vial
			2 x 0.2 ml	NEFA	Freeze 2 x 0.2 ml	
			0.2 ml	β-hydroxybutyrate	Freeze 0.2 ml	
			0.2 ml	Leptin	Freeze 0.2 ml	
			0.2 ml	Adiponectin	Freeze 0.2 ml	
			3 x 0.2 ml	Klotho	Freeze 3 aliquots	Send to Prof. Sandrine at Institute of Psychiatry, KCH

Appendix 12 - International physical activity questionnaire – short form (IPAQ-SF) given to participants of the Met-IER study.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

   _____ **days per week**

   ☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

   _____ **hours per day**

   _____ **minutes per day**

   ☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

   _____ **days per week**

   ☐ No moderate physical activities → ***Skip to question 5***

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

Appendix 13 - Participant's booklet on healthy eating based on a Mediterranean-type diet and individually personalised dietary and physical activity goals.

KING'S College LONDON

Participant's Booklet

Healthy Eating

A healthy balanced diet

It is important to enjoy a variety of foods from all of the different food groups to enable you to become healthier and help manage your weight.

The Eatwell plate

The Eatwell plate shows the proportions of the main food groups that make up a healthy diet. It shows what a well balanced healthy diet should look like over a day or a week.

Your diet should be based on starchy foods such as potatoes, bread, rice and pasta, along with plenty of fruit and vegetables, protein rich foods such as meat, fish, eggs, beans and lentils, milk and dairy foods and limited fat, sugar and salt.

For weight loss - Fill half of your plate with vegetables/salad, then a quarter of your plate with protein sources such as meat, fish, eggs or beans and the other quarter with starchy foods like potatoes, rice, pasta or bread at each meal.

Healthy eating tips

- ✓ **Base your meals on starchy foods** such as bread, cereals, chapattis, potatoes, rice, noodles and pasta. These provide energy along with calcium, iron, B-vitamins and fibre. Choose wholegrain and wholemeal varieties.
- ✓ **Eat plenty of fruit and vegetables-** These are low in calories and provide vitamins, minerals (folate, vitamin C, potassium) fibre and antioxidants. Aim to have at least 5 portions which can include fresh, frozen, dried, canned and juices. Be aware that juice, smoothies and dried fruit can be high in energy.
- ✓ M**ilk and dairy-** These provide calcium, protein and vitamins. Aim for 2-3 portions per day of low fat versions such as semi skimmed/skimmed milk, lower fat hard cheeses/cottage cheese and low fat yoghurts.
- ✓ **Meat, fish, eggs, beans and other non dairy** - These provide an important source of protein. Aim for 2-3 portions per day.
- ✓ **Eat more fish-** Aim to have at least 2 portions of fish per week. One portion should be oily such as salmon, mackerel, herring, trout, sardines, kippers or fresh tuna as these contain omega 3 which may help to prevent heart disease. Omega 3 rich alternatives include green leafy vegetables, soy, canola oil, rapeseed oil, walnuts, flaxseeds and fortified foods.
- ✓ **Cut down on saturated fat, sugar and processed foods-** Limit saturated fats found in fats at rooms temperature such as butter, high fat snacks such as biscuits, sauces, processed meats and takeaways. Limit red meat to 1-2 per week. Instead choose skinless lean cuts of white meat such as chicken or turkey and remove any excess fat. Instead choose unsaturated fats such as vegetable oils e.g. olive oil, rapeseed oil and sunflower oil. However portion sizes should be kept to 1tsp per meal as all fats are high in calories. Sugary foods should also be limited as they are high in energy and contribute to tooth decay.
- ✓ **Eat less salt -** Aim for less than 6g per day (approx. 1 tsp) as salt can increase your blood pressure. 75% of the salt we eat is from ready made foods we buy so reduce processed meat, takeaways, ready meals, soups, packet sauces, gravy, stock cubes and salty snacks. Use little or no salt in cooking and limit adding salt at the table. Instead use herbs, spices and garlic.
- ✓ **Choose suitable cooking methods-** Avoid frying, instead bake, boil, steam or microwave.
- ✓ **Keep hydrated-** Aim to drink 8-10 glasses of fluid per day. Water is the best choice or sugar free squash and diet drinks in moderation.
- ✓ **Maintain a regular meal pattern** and do not skip meals.
- ✓ **Increase fibre** by increasing beans, pulses, lentils, oats, fruits and vegetables, oat bran, linseeds and chickpeas as these make you feel fuller for longer.
- ✓ **Keep active –** aim for 30 minutes of moderate exercise (enough to raise heart rate) at least 5 times per week. Try to be more active within your daily life such as getting off the bus one stop early and walking, taking the stairs and using a pedometer to gradually build up to approximately 10,000 steps /day.

Practical portion sizes

A portion of potato (baked or mashed) should be about the size of a computer mouse or two eggs

A serving of cheese should be the size of a matchbox

Fill your cupped hand with nuts or pretzels to see one portion

Use the tip of your thumb as a guide for how much oil to use

A serving of meat or fish should be about the size of a deck of cards or the palm of your hand

One cupful is the right amount of cooked starchy food such as pasta and rice – this is about the size of a tennis ball

Further Tips:

- ✓ Measure out specified portion sizes using the same scales, cup or plate so that you can soon see the appropriate portion size.
- ✓ Measure out one serving and then put it away.
- ✓ Eat off a smaller plate.
- ✓ Check the recommended portion sizes and food labels.
- ✓ You do not have to finish all of the food on your plate. Stop when full and put left overs straight into the fridge or freezer.

Practical tips and minimising food cravings

- ✓ Eat slowly to avoid overeating as it takes 15-20 mins for our brains to register that we are full.
- ✓ Focus on your food without other distractions to avoid overeating.
- ✓ Plan ahead- Take a healthy packed lunch when you are out and about and healthier snacks to have on the go. Cook healthier meals in bulk and freeze individual portions.
- ✓ Keep a food diary to stay aware of what you are eating and any emotional triggers. This can help you to keep in control of your eating.
- ✓ Make a list of non-food related tasks that will distract you from thinking about eating for example reading a book or going for a walk.
- ✓ Get support from others family/friends

Healthy food swaps

Swap:	For:
Biscuits, sweets or chocolate	Fresh fruit, dried fruit or nuts
Whole milk	Semi- skimmed or 1% fat milk
Sugar coated breakfast cereal	Wholegrain cereal e.g. porridge, wholegrain wheat, weetabix.
Sugar on breakfast cereal	Fresh or dried fruit
Full fat yoghurts	Natural low fat yogurt, fat free yoghurt.
White bread, pasta, bagels and muffins	Wholegrain and wholemeal varieties
Butter and cheese on a baked potato	Reduced fat spread and reduced salt and sugar baked beans.
Tuna melt sandwich	Tuna salad sandwich without mayonnaise
Cream sauce or cheese sauces	Vegetable based sauces
Mashed potato made with butter/ whole milk	Mashed potato made with low fat spread or a lower fat milk.
Fizzy drinks, Fruit squash	Water with no added sugar
Hot chocolate	Low calorie hot chocolate e.g. Cadbury highlights, options.

Remember small changes make a big difference!

Alcohol

- Guidelines for men and women state that you should not drink regularly more than 14 units per week and no more than 2-3 units per day.
- Be aware that alcohol is high in calories so can lead to weight gain:
- 1 unit provides 56 kcal and can stimulate appetite.
- Tips- Drink spritzers, set limits, choose low calorie mixers and smaller glasses.

Food labelling

- Show the amount in a portion of food or per 100g.
- Colours green, amber, red show if a food is low (green) medium (amber) or high (red) for the amount of energy or nutrients in a portion.
- Be aware that the portion sizes may not be the portion you are eating.
- Choose mainly green and amber labels and limit red labels.

All measures per 100g	**Low** A healthier choice	**Medium** OK most of the time	**High** Just occasionally
Sugars	5g or less	5.1g – 22.5g	More than 22.5g
Fat	3g or less	3.1g – 17.5g	More than 17.5g
Saturates	1.5g or less	1.6g – 5g	More than 5g
Salt	0.30g or less	0.31g – 1.5g	More than 1.5g

Tips for eating out:

- ✓ Try to plan what you are going to eat beforehand and look out for menu choices labelled as healthy options.
- ✓ Choose "skinny" drinks made with skimmed milk.
- ✓ Choose scone/bun instead of pastry, cream or chocolate cake.
- ✓ Avoid extra cheese, mayonnaise and creamy sauces.
- ✓ You could share a starter or dessert.
- ✓ Ask for dressings and dips to be on the side so you can control the portion sizes.
- ✓ Choose vegetable based soups and sauces and avoid cream, cheese or coconut based sauces.
- ✓ Choose steamed or stir-fried dishes and not deep fried.
- ✓ Choose plain boiled rice instead of fried rice and boiled jacket potato rather than chips or wedges.

Further information:

BDA food factsheets: https://www.bda.uk.com/foodfacts/home.
Change4life: http://www.nhs.uk/chang4life/pages/healthy-eating.aspx
Eat well plate: http://www.nhs.uk/Livewell/Goodfood/Pages/eatwell-plate.aspx
Weight loss guide:https://www.bhf.org.uk/publications/healthy-eating-and-drinking/facts-not-fads---your-simple-guide-to-healthy-weight-loss
Portion size guide: https://www.bhf.org.uk/publications/healthy-eating-and-drinking/eat-better-feel-better---your-bite-sized-guide-to-health-weight-loss
Alcohol advice: Drinkware: https://www.drinkaware.co.uk

Dietary goals

Goal 1	
Goal 2	
Goal 3	
Goal 4	
Activity Goal	

Appendix 14 - Nutritional composition of the LighterLife® foodpacks provided to the participants of the Met-IER study.

Milkshakes per serving

	Banana		Chocolate		Caramel		Strawberry		Vanilla	
	40g	% RI	42g	% RI	42g	% RI	40g	% RI	40g	% RI
Energy (kJ)	643		627		633		639		638	
(kcal)	153		150		151		152		152	
Fat (g)	4.6		3.7		3.9		4.6		4.6	
Saturates fat (g)	1.9		1.4		1.4		1.9		1.9	
Carbohydrates (g)	12.7		12.6		12.5		12.7		12.5	
Sugars (g)	11.9		4.9		5.0		11.4		11.8	
Fibre (g)	3.6		7.4		7.3		3.8		3.9	
Protein (g)	13.4		12.9		12.9		13.1		13.2	
Salt (g)	1.4		1.2		1.2		1.4		1.4	
Vitamin A (µg)	200	25	259	32	261	33	200	25	200	25
Vitamin D (µg)	1.25	25	1.7	34	1.7	34	1.25	25	1.25	25
Vitamin E (mg)	3	25	4.3	36	4.6	39	3	25	3	25
Vitamin K (µg)	25	33	32.2	43	32.2	43	25	33	25	33
Vitamin C (mg)	20	25	22.5	28	22.5	28	20	25	20	25
Thiamin (mg)	0.28	25	0.34	30	0.4	32	0.28	25	0.28	25
Riboflavin (mg)	0.4	29	0.5	36	0.5	36	0.4	29	0.4	29
Niacin (mg)	4.9	31	5.7	36	5.7	36	4.9	31	4.9	31
Vitamin B6 (mg)	0.5	36	0.6	46	0.6	46	0.5	36	0.5	36
Folic acid (µg)	50	25	50	25	63.4	32	50	25	50	25
Vitamin B12 (µg)	0.6	25	0.9	37	0.9	37	0.6	25	0.6	25
Biotin (µg)	12.5	25	14	28	15.5	31	12.5	25	12.5	25
Pantothenic acid (mg)	1.5	25	1.9	32	1.9	32	1.5	25	1.5	25
Potassium (mg)	540	27	547	27	531	27	527	26	534	27
Chloride (mg)	243	30	249	31	204	26	235	29	239	30
Calcium (mg)	288	36	213	27	201	25	277	35	283	35
Phosphorus (mg)	288	41	231	33	193	28	280	40	284	41
Magnesium (mg)	105	28	106	28	93.8	25	104	28	104	28
Iron (mg)	4.0	29	5.6	40	4.8	34	4.0	29	4	29
Zinc (mg)	2.5	25	3.2	32	3.0	30	2.5	25	2.5	25
Copper (mg)	0.38	38	0.55	55	0.4	43	0.38	38	0.38	38
Manganese (mg)	0.55	28	0.65	32	0.7	32	0.55	28	0.55	28
Fluoride (mg)	0.88	25	1.1	32	1.1	32.	0.88	25	0.88	25
Selenium (µg)	13.8	25	17.8	32	17.8	32	13.8	25	13.8	25
Chromium (µg)	10	25	15.7	39	12.9	32	10	25	10	25
Molybdenum (µg)	12.5	25	16.1	32	16.1	32	12.5	25	12.5	25
Iodine (µg)	37.5	25	48.5	32	48.5	32	37.5	25	37.5	25

Other foodpacks per serving

	Soup		Spaghetti Bolognese		Curry		Bars	
	40g	% RI	40g	% RI	40g	% RI	46g	% RI
Energy (kJ)	633		630		634		652	
(kcal)	150		150		151		155	
Fat (g)	5.0		4.2		4.5		4.5	
Saturates fat (g)	1.6		0.6		1.9		1.9	
Carbohydrates (g)	12.7		13.9		12.5		16.9	
Sugars (g)	4.8		2.0		4.1		9.5	
Fibre (g)	2.5		3.2		4.3		3.8	
Protein (g)	12.5		12.5		12.7		12.7	
Salt (g)	1.5		1.4		0.9		0.3	
Vitamin A (µg)	245	31	204	26	200	25	204	25
Vitamin D (µg)	1.8	36	1.3	25	1.3	25	1.3	25
Vitamin E (mg)	3.0	25	3.3	27	3.0	25	3.0	25
Vitamin K (µg)	29.8	40	25	34	25	33	25	34
Vitamin C (mg)	29.1	37	30.6	38	20	25	20	25
Thiamin (mg)	0.3	30	0.32	29	0.3	28	0.3	25
Riboflavin (mg)	0.5	38	0..44	31	0.4	30	0.4	29
Niacin (mg)	4.9	31	4.8	30	5.0	31	4.6	28
Vitamin B6 (mg)	0.6	40	0.6	39	0.5	37	0.5	36
Folic acid (µg)	130	65	195	98	68.7	34	51	25
Vitamin B12 (µg)	0.9	36	0.6	25	0.6	25	0.7	26
Biotin (µg)	14.1	28	12.5	25	12.5	25	13	25
Pantothenic acid (mg)	1.8	30	1.5	26	1.6	26	1.6	25
Potassium (mg)	830	42	525	27	501	25	536	27
Chloride (mg)	611	77	627	78	392	49	201	25
Calcium (mg)	200	25	200	25	238	30	208	26
Phosphorus (mg)	286	41	191	28	188	27	177	25
Magnesium (mg)	104	28	98	26	96	25	95	25
Iron (mg)	5.0	36	4.6	33	5.0	35	4.9	35
Zinc (mg)	2.7	27	2.9	29	2.8	28	2.8	28
Copper (mg)	0.4	42	0.3	31	0.5	48	0.5	45
Manganese (mg)	0.5	27	0.5	26	0.6	28	0.6	30
Fluoride (mg)	0.9	25	0.9	25	0.9	25	0.9	25
Selenium (µg)	14.7	27	13.9	25	13.8	25	14	25
Chromium (µg)	11.1	28	10	25	10	25	10	25
Molybdenum (µg)	18.7	38	12.5	25	12.5	25	13	25
Iodine (µg)	37.8	25	37.5	25	37.5	25	38.5	25